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14. ABSTRACT

ETS factors comprise a large transcription factor family known to play a significant role in cellular development, differentiation, and transformation. Emerging evidence reveals that increased mRNA expression of the human Ets factor-1, ESE-1, is associated with breast cancer. Stable expression of ESE-1 transforms MCF-12A immortalized human mammary epithelial cells. However, little is known about ESE-1 protein expression and its role in maintaining the transformed phenotype in human breast cancer cell lines. Here, we used an anti-ESE-1 mouse monoclonal antibody in Western blot and immunofluorescent cell analyses to show that ESE-1 is expressed as a nuclear protein in MCF-7, T47D and ZR-75 transformed, tumorigenic mammary epithelial cell lines, and that it is not expressed in transformed MDA-MB-231 and nontransformed MCF-10A and MCF-12A cells. In addition, specific knockdown of endogenous ESE-1 in the human breast carcinoma ZR-75 and MCF-7 cell lines decreased colony formation and anchorage independent growth. Mechanistically, ESE-1 knockdown decreased cellular proliferation, but had no effect on apoptosis. Finally, serum withdrawal resulted in a time-dependent, ~90% reduction of ESE-1 protein production in MCF-7 cells. These results establish that ESE-1 plays a key role in maintaining the transformed phenotype in breast cancer, thus providing a novel single-point target for breast cancer therapy.

15. SUBJECT TERMS
ETS, Ese-1, Esx, MCF-12A, transformation, mammary, breast cancer

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Table of Contents

INTRODUCTION

The Ets family of transcription factors contains several members that are important components of the cellular pathways leading to tumorigenesis (1). For example, several Ets members are downstream targets of oncogenic Ras (2); dominant-negative Ets reverses the transformed phenotype (3,4); and, Ets proteins have been shown to regulate a repertoire of genes that govern cellular survival, proliferation and migration (1,6). Moreover, several Ets factors have been implicated in breast cancer (1,6). However, the ability of Ets factors to transform human breast cells, the identity of the precise Ets factor required for breast cell transformation, and the molecular mechanism by which such an Ets factor mediates breast cell transformation, all remain unknown. The ESE-1/ESX gene is an Ets member that is particularly relevant to breast cancer. ESE-1 is located on chromosome 1q32.1, in a region that is amplified in 50% of early breast cancers. ESE-1 mRNA is over-expressed in human breast ductal carcinoma in situ (DCIS) (7-9). Also, there is a positive feedback loop between the HER2/neu proto-oncogene and ESE-1, in that HER2/neu activation induces ESE-1 expression, while ESE-1 activates the HER2/neu promoter via a putative ESX DNA binding site (7-9). Finally, HER2/neu and ESE-1 expression levels are positively correlated in human breast cancer cell lines (7-9). ESE-1 is expressed in both nuclear and cytoplamic compartments. We discovered that ESE-1 transforms MCF-12A cells via a novel cytoplasmic mechanism in which a unique 40-amino acid (AA), serine- and aspartic acid rich (SAR) domain is necessary and sufficient for transformation (10-12). Furthermore, we reported that ESE-1 protein is abundantly expressed in the cytoplasm of human ductal carcinoma in situ (DCIS) specimens (12). However, identification of the cytoplasmic partners of the ESE-1 SAR motif and the precise mechanism by which cytoplasmic signaling mediated by the ESE-1 SAR motif occurs remain unknown. Current data reveals that this 40-AA SAR domain functions autonomously, indicating that this motif is sufficient to recognize and activate cytoplasmic partner proteins, thus transforming MCF-12A human mammary epithelial cells (12). Our original goal was to apply innovative and powerful state-of-the-art molecular yeast two-hybrid and protein biophysical methods (MALDI-TOF and LC-mass spectrometry) to identify proteins that interact with the ESE-1 SAR transformation domain.

While we spent a considerable amount of time and effort pursuing these studies, we have not yet been able to unambiguously identify a cytoplasmic protein that is the key functional ESE-1 interacting partner mediating the transformed phenotype. Nevertheless, we remain committed to these studies, and we plan to pursue them as Aim 4 in this final grant year. But in order to maintain strong productivity, we shifted our research to a more productive avenue of investigation. Thus, we set out to define whether endogenous ESE-1 is required to maintain the transformed phenotype. To this end, we established three new aims (Aims 1-3). We have discovered that endogenous ESE-1 is required to maintain the transformed phenotype in two breast cancer cell lines, via a proliferation mechanism. We also just submitted a paper for publication, which is included as a PDF in this report (Walker et al, 2008). Below is a summary of these new results.

ETS factors comprise a large transcription factor family known to play a significant role in cellular development, differentiation, and transformation. Emerging evidence reveals that increased mRNA expression of the human Ets factor-1, ESE-1, is associated with breast cancer. Stable expression of ESE-1 transforms MCF-12A immortalized human mammary epithelial cells. However, little is known about ESE-1 protein expression and its role in maintaining the transformed phenotype in human breast cancer cell lines. We used an anti-ESE-1 mouse monoclonal antibody in Western blot and immunofluorescent cell analyses to show that ESE-1 is expressed as a nuclear protein in

MCF-7, T47D and ZR-75 transformed, tumorigenic mammary epithelial cell lines, and that it is not expressed in transformed MDA-MB-231 and nontransformed MCF-10A and MCF-12A cells. In addition, specific knockdown of endogenous ESE-1 in the human breast carcinoma ZR-75 and MCF-7 cell lines decreased colony formation and anchorage independent growth. Mechanistically, ESE-1 knockdown decreased cellular proliferation, but had no effect on apoptosis. Finally, serum withdrawal resulted in a time-dependent, ~90% reduction of ESE-1 protein production in MCF-7 cells. These results establish that ESE-1 plays a key role in maintaining the transformed phenotype in breast cancer, thus providing a novel single-point target for breast cancer therapy.

BODY

Task 1: To characterize ESE-1 expression in human breast cancer cells and to identify shRNAs capable of knocking-down endogenous ESE-1.

a) Untransformed MCF-10A and MCF-12A cells fail to express endogenous ESE-1, whereas MCF-7, T47D and ZR-75 breast cancer cell lines express ESE-1.

Using a small-grant support mechanism provided by our Cancer Center, we developed several novel, high-affinity, high-specificity murine monoclonal antibodies targeting amino acids 128-259, spanning the transcription activation domain (TAD), SAR, and AT-hook domains of human ESE-1 protein. The manuscript describing the generation and characterization of these antibodies is currently in preparation. For the studies in this report, we used one of these antibodies: anti-ESE-1 mAB405. Western blot analysis of whole cell extracts probing for endogenous ESE-1 was performed on a series of human transformed and nontransformed mammary epithelial cell lines and compared with qRT-PCR. As shown in Fig. 1, ESE-1 protein was not detected by Western blot analysis in the nontransformed MCF-10A and MCF-12A mammary epithelial cells lines. In contrast, ESE-1 protein was detected in the tumorigenic MCF-7, T47D and ZR-75 cell lines, with the levels in MCF-7 and T47D being equivalent and greater than that expressed in ZR-75. Noteworthy, ESE-1 protein was not detectable in the highly metastatic MDA-MB-231 cells (Fig. 1A). Quantitative RT-PCR mRNA analysis validated these protein data (data not shown). To further confirm ESE-1 protein expression levels and its subcellular localization, we performed indirect immunofluorescence cytochemistry (ICC) studies, with cell nuclei counterstained with DAPI to define each cell (Fig. 2). These data revealed that MCF-10A, MCF-12A and MDA-MB-231 cells fail to express any ESE-1 protein detectable by this ICC method, whereas endogenous ESE-1 protein was detected in transformed MCF-7, T47D

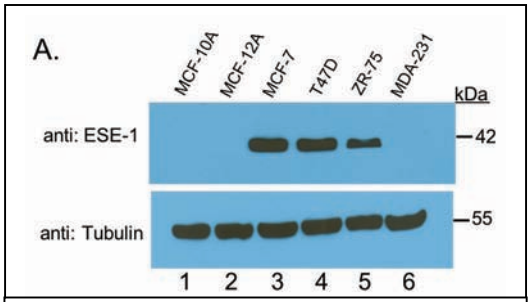

Fig. 1: Western blot of mammary cell whole cell lysates. Anti-Ese-1 (top) and anti-tubulin (bottom).

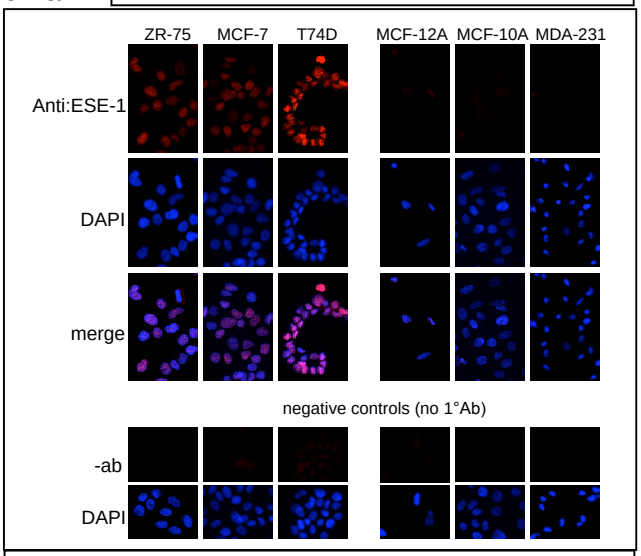

Fig. 2: ICC analysis of ESE-1 subcellular localization in breast cancer cells. Anti-Ese-1 (red), DAPI and Merge (purple). Negative controls shown in bottom panel.

and ZR-75, and in each case ESE-1 was localized to the nucleus (Fig. 2). In general, the ICC data further confirmed the Western blot data and revealed ESE-1 protein to be localized in the nucleus.

b) *shRNA targeting of ESE-1 knocks down endogenous ESE-1 protein expression*.

In initial optimization studies of several shESE-1 constructs, we identified an shESE-1 construct (shESE-1), which targeted the ETS DBD of ESE-1, that optimally knocked down endogenous ESE-1 (data not shown). Using shESE-1, we established the time course of ESE-1 knockdown by transiently transfecting ZR-75 cells with shESE-1 and preparing whole cell lysates 2, 3, 4 and 5 days post-transfection (Fig. 3A). As a control, we transfected cells with an shRNA empty vector control (vector) and prepared whole cell lysates 2 days post-transfection (Fig. 3A). The whole cell lysates were then probed for ESE-1 and tubulin by Western blot analysis (Fig. 3A). These results show that compared to vector control, essentially a complete knockdown of ESE-1 occurs by 2 days, and a significant reduction of ESE-1 persists up to 5 days post-transfection of shESE-1 (Fig. 3A). The tubulin control shows that a nearly equivalent amount of protein was loaded in each lane. A similar time course of shESE-1 knockdown was performed in MCF-7 cells and this study showed the same complete reduction of ESE-1 by 2 days, but in MCF-7 cells, this strong level of inhibition persisted up to 5 days (data not shown). Next, we sought to establish the specificity of ESE-1 knockdown. As noted above, this shESE-1 targeted the ETS DBD, which is conserved amongst ETS proteins. Computational analysis of the shESE-1 target sequence revealed it to be unique to ESE-1, with our target sequence showing minimal similarity only to ETS-1/ETS-2 (with only 4 of 19 nt being identical for each). As a negative control (shCtr), we used an shRNA construct that also targeted the ESE-1 ETS DBD, but which in optimization studies failed to inhibit ESE-1 expression. We transiently transfected MCF-7 cells with shCtr and shESE-1 shRNA vectors, prepared whole cell lysates 2 days post-transfection, and probed for ESE-1, Ets-1/Ets-2 and tubulin by Western blot analysis (Fig. 3B). This study reveals that the shCtr failed to inhibit endogenous ESE-1, while the shESE-1 vector resulted in a robust knockdown of ESE-1 in MCF-7 cells. We quantitated this inhibition by normalizing ESE-1 expression to tubulin and found that ESE-1 expression is reduced ~4-fold in the shESE-1 cells compared to shCtr. Finally, to determine the specificity of shESE-1 knockdown, we performed Western blot analysis for both ETS-1 and ETS-2, using an antibody that recognizes both ETS factors. As shown in Fig. 3B, neither the shCtr nor shESE-1 affected the levels of ETS-1 plus ETS-2, affirming shESE-1's specificity to knockdown endogenous ESE-1, and that the shCtr failed to inhibit ESE-1, ETS-1 and ETS-2.

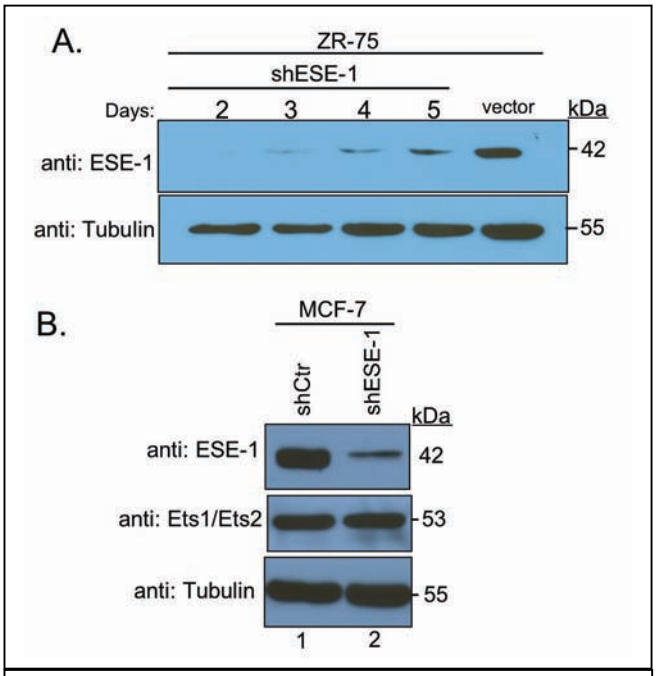

Fig. 3: Time-course of shEse-1 knock-down in ZR-75 and specificity of knockdown in MCF-7 breast cancer cells. Cell lysates prepared after 2-5 days post shESE-1 transfection were analyzed for ESE-1 and tubulin in ZR-75 cells (A); or 2-days post-transfection and blotted for ESE-1, Ets-1/2 and tubulin in MCF-7 cells (B).

Task 2: To determine whether ESE-1 knockdown in human breast cancer cell lines reverses the transformed phenotype.

a) *Knockdown of ESE-1 reduces the clonal cell growth and soft agar colony-forming ability of ZR-75 and MCF-7 cells*

In order to determine if ESE-1 was necessary for colony formation, ZR-75 and MCF-7 cells were each co-transfected with pEGFP-C3, to confer G418 resistance, and shCtr or shESE-1. Transfected cells were selected with G-418 for 14 days, resultant colonies were stained with Crystal violet, and counted by direct visualization. Shown are the results of the MCF-7 transfection revealing ~27 colonies per shCtr plate and only ~6 colonies formed in the presence of shESE-1, resulting in a 5.5-fold reduction in MCF-7 colony formation (Fig. 4A). Transfection of ZR-75 cells yielded ~43 and 3 cells with shCtr and shESE-1, respectively, resulting in a 13-fold reduction in ZR-75 colony formation (data not shown). Of note, several attempts to generate stable ESE-1 knockdown cell lines resulted in few, small colonies that failed to grow, precluding clonal expansion. Importantly, the similar inhibitory effect of ESE-1 knockdown on colony formation in two distinct breast cancer cell lines supports the critical role of ESE-1 in the growth of transformed mammary cells.

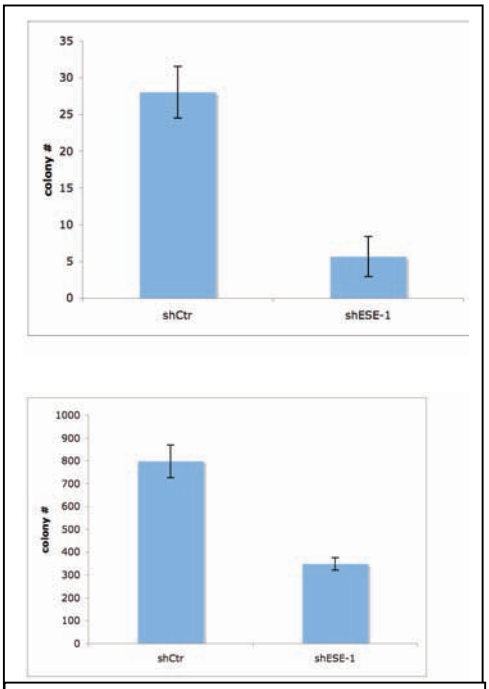

Fig. 4: Clonal cell growth (A) and soft agar colony formation (B) in MCF-7 cells post shCtr and shESE-1 transfection.

To further investigate the functional role of ESE-1 in the tumorigenic phenotype, we performed similar shRNA knockdown studies of ESE-1 and performed soft agar experiments using MCF-7 cells. We chose to focus on MCF-7 cells for these soft agar experiments, because MCF-7 cells yielded larger colonies transient transfection resulted in a more effective and prolonged knockdown of endogenous ESE-1 (data not shown). This more prolonged knockdown after MCF-7 transient transfection is important, since the soft agar assay is over 14 days and we could not generate stable shESE-1 knockdown cells to then plate in soft agar. Because in the transient transfection approach not all cells are transfected and ESE-1 expression is likely to re-appear at later time points, compared to G418 selection methods, the resulting colony number in the shESE-1 knockdown cells presented here is likely an overestimate. Thus, we transiently transfected MCF-7 cells with shCtr or shESE-1 vector DNAs, plated the cells in soft agar and after 14 days counted the colonies growing in an anchorage independent manner (Fig. 4B). MCF-7 cells transfected with shCtr generated ~800 colonies, whereas shESE-1-transfected cells generated ~350 colonies, a 56% reduction in colony formation (Fig. 4b, plated in sextuplicate). A separate study showed that shESE-1 mediated a 64% reduction in MCF-7 soft agar colony number, with MCF-7 cells transiently transfected with shCTR- and shESE-1 yielding 575 and 209 soft agar colonies, respectively (data not shown). Cloning efficiency was determined by dividing the number of cells seeded by the number of colonies formed times 100. Control cells yielded a cloning efficiency of 1.6%, while shESE-1 cells had a cloning efficiency of 0.7%. Thus, taken together, these assays show that ESE-1 is required to maintain the tumorigenic phenotype of MCF-7 cells.

Task 3: To determine the molecular mechanism of by which knock-down of endogenous ESE-1 regulates the transformed phenotype.

a) *Reversion of the Transformed Phenotype is not due to Apoptosis*

Having demonstrated a reduction in colony formation and anchorage independent growth, we next sought to address the mechanism responsible for the reversion of the malignant phenotype. We first tested whether knockdown of ESE-1 in MCF-7 cells resulted in apoptosis. In order to address this point, MCF-7 cells were transiently transfected with shCtr or shESE-1, and harvested at 48 and 72 hours. DNA laddering assay failed to show any shESE-1 induced apoptosis (data not shown). To further investigate apoptosis as a biological response to ESE-1 knockdown, we analyzed caspase 3 and/or 7 activation, using a luminescent enzyme activity assay (CaspaseGlo 3/7 Assay, Promega). With the data set to 1 for untransfected controls, these results show that there is no change in caspase 3/7 activity in shESE-1 transfected cells, compared to empty vector and shCtr transfected cells, at either the 48 (data not shown) or 72 hour time points (Fig. 5). Having excluded apoptosis as the cellular mechanism responsible for the shESE-1-induced inhibition of colony formation, we next sought to determine whether shESE-1 affected MCF-7 cellular proliferation.

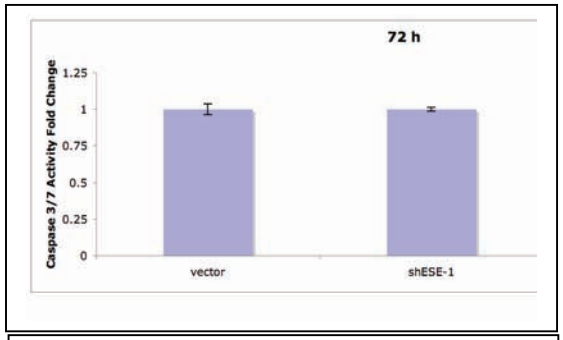

Fig. 5: Caspase 3/7 activity in MCF-7 cells 72-hr post shCtr and shESE-1 transfection.

b) *Reversion of the Transformed Phenotype is due to shESE-1-mediated Inhibition of MCF-7 Cell Proliferation*

The control of cellular proliferation is a key mechanism in the prevention of tumorgenicity and malignancy. ESE-1's ability to transcriptionally regulate the growth-promoting *Her2/neu* and *TGF-βRII* receptors genes suggests that it has an important role in controlling cellular proliferation. In order to confirm ESE-1's role in maintaining cellular proliferation in MCF-7 transformed cells, MCF-7 cells were transiently transfected with shCtr or shESE-1, and cellular proliferation was determined at 2-, 4- and 6-days post-transfection by counting total viable cells (Fig. 6). A representative total cell proliferation study, performed in duplicate, shows that shESE-1 cells display significantly reduced proliferation at each time point (Fig. 6). At the start of the study, 50,000 cells were plated, and the results reveal a 62%, 78% and 66% reduction in cell proliferation at 2- 4- and 6-days, respectively, in the shESE-1 knockdown cells compared to shCtr cells (Fig. 6). Similar results were obtained using an MTS proliferation assay, which showed a 1.6 fold (or ~62%) reduction in MTS absorbance at 6-days post-transfection in the shESE-1 transiently transfected MCF-7 cells, compared to shCtr control cells (data not shown). These data demonstrate that ESE-1 is required for optimal MCF-7 cellular proliferation and reveal the mechanism by which ESE-1 contributes to the transformed phenotype.

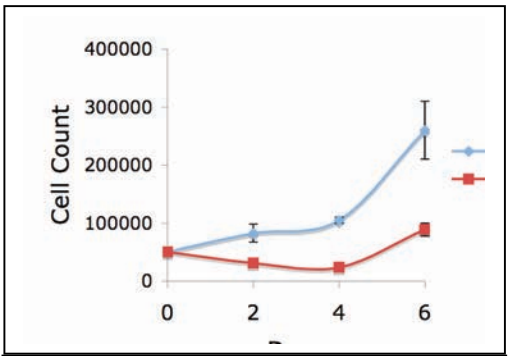

Fig. 6: Cell growth of MCF-7 cells (50000) 2, 4 and 6 days post-transfection with shCtr (blue) or shESE-1 (red).

c) *Serum starvation extinguishes ESE-1 protein expression in MCF-7 cells*

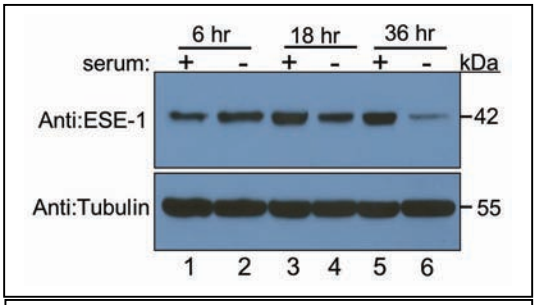

Fig. 7: Extinction of ESE-1 expression in MCF-7 cells upon serum withdrawal. Cells plated in (+) or (-) serum, and lysates prepared 6,18 and 36 hrs later and analyzed by WB for ESE-1 (top) and tubulin (bottom).

Having shown that MCF-7 cells express endogenous ESE-1 protein (Fig. 1) and that ESE-1 expression is required to maintain the MCF-7 transformed phenotype by controlling cellular proliferation (Figs. 4-6), we sought to determine whether ESE-1 protein production was growth factor dependent. This question is relevant because ESE-1 protein regulates *HER2/Neu* promoter activity (10) and HER2/Neu has been shown to activate the *ESE-1* promoter, suggesting that growth factors present in serum may play a critical role in regulating *ESE-1* gene expression and ESE-1-mediated mammary cell proliferation. Here, we grew MCF-7 cells in either complete (10% FBS) or in serum-depleted (0.1% FBS) media for 6, 18 and 36 hrs, and then probed for ESE-1 and tubulin protein expression by Western blot analysis of whole cell extracts. As shown in Fig. 7, ESE-1 protein appears to increase slightly at the 6 hr post-starvation time-point, only to decrease progressively at the 18 and 36 hr post-starvation time-points, compared to complete media controls. Quantitation of the 36 hr post-starvation time-point revealed a 90% reduction of ESE-1 protein expression, when normalized to tubulin and compared to the 36 hr complete media control. The Western blot for tubulin reveals that protein loading was equivalent in all lanes. These data raise the interesting possibility that reduction in MCF-7 cell proliferation due to serum starvation may be due, at least in part, to reduced ESE-1 protein expression.

Task 4: To identify cytoplasmic and nuclear ESE-1-interacting proteins.

a) *Identification of SAR-interacting cytoplasmic protein(s) and ESE-1-binding nuclear factors*

This was the original goal of this project. This study has been frustratingly difficult, since we've applied SAR-affinity purification, yeast 2-hybrid and co-IP approaches to no avail. However, we are re-energized now that we have generated two new high-affinity, high-specific monoclonal antibodies targeting distinct ESE-1 domains, both of which IP endogenous ESE-1. Further, we've begun to optimize an avidin/biotin magnetic bead-based purification method that should yield co-IPs with much less contaminants. Thus, in the final grant year, we plan to use our Flag epitope-tagged Flag-GFP-NES-SAR constructs, targeting SAR expression exclusively to the cytoplasm, and use a combination of biotin-labeled anti-Flag and/or anti-ESE mAbs and avidin-magnetic beads to purify cytoplasmic SAR-interacting proteins. Using a similar approach, targeting Flag-GFP-intact ESE to the nucleus, will be used to co-IP nuclear ESE-1-binding partner proteins. Finally, our Mass spec collaborator, Dr. Kirk Hansen, has optimized in-gel and solution based MALDI methods.

KEY RESEARCH ACCOMPLISHEMENTS

- Generation of shESE-1 constructs that successfully knock-down endogenous human ESE-1.
- Identification of endogenous ESE-1 as being required to maintain the transformed phenotype of MCF-7 and ZR-75 human mammary epithelial cells.
- Demonstration that the mechanism by which ESE-1 maintains the transformed phenotype is due to its requirement for cell proliferation.
- ESE-1 appears to contribute to the serum-induced proliferative response in breast cancer cells.

REPORTABLE OUTCOMES

Abstracts:

1. Walker DM, Pozcobutt J, Gonzales MS, Horita H, **Gutierrez-Hartmann A**. ESE-1 is required to maintain the transformed phenotype of MCF-7 and ZR-75 human mammary epithelial cells. Era of Hope Meeting, Baltimore, MD, June, 2008.

Manuscripts

1. **Gutierrez-Hartmann A**, Duval DL, Bradford AP. ETS transcription factors in endocrine systems. *Trends Endocrinol Metab*, 18(4): 150-158, 2007.
2. Jedlicka P, **Gutierrez-Hartmann A**. ETS transcription factors in intestinal morphogenesis and disease. *Histology and Histopathology*. Invited review, *in press*.
3. Walker DM, Pozcobutt J, Gonzales MS, Horita H, **Gutierrez-Hartmann A**. ESE-1 is required to maintain the transformed phenotype of MCF-7 and ZR-75 human mammary epithelial cells. *Submitted*.
4. Prescott JD, Gonzales M, **Gutierrez-Hartmann A**. Nuclear export and phosphorylation target sequences in ESE-1 mediate transformation of MCF-12A human mammary epithelial cells. *In preparation*.

Reagents Developed

1. shEse-1 knock-down vectors.
2. We also generated a number of reagents relevant to our original aims, including a number of Flag epitope-tagged SAR and ESE-1 constructs.

Doctoral training

1. Darius Walker, an URM working on this project, completed his PhD thesis studies in the Molecular Biology Graduate Program.

Awards

1. Arthur Gutierrez-Hartmann, PI, was selected for the prestigious 2008 AACR-Minorities in Cancer Research - Jane Cooke Wright Lectureship Award.

New Grants

1. I plan to use the data generated with this DOD support to write new grants applications for NIH/NCI (Fall, 2008), Komen Foundation (August, 2008), Mary Kay Ash Foundation (Fall, 2008), and DOD Idea (Spring, 2009) support mechanisms.

CONCLUSIONS

The ETS transcription factor family is known to play a significant role in many cancers, with aberrant expression of ESE-1 being correlated in nearly 50% of early human breast tumors. Previously, we have shown that stably expressed HA-ESE-1 or GFP-ESE-1 imposes the transformed phenotype on nontransfromed, ESE-1-negative MCF-10A and MCF-12A cells (10-12), and that cytoplasmic localization of a 40-AA SAR domain is necessary and sufficient to mediate this effect (12). Consistent with this conclusion, PAK-1-mediated phosphorylation of the SAR domain of exogenous ESE-1 in the cytoplasm modulates stability and transformation potency of ESE-1 in ZR-75 cells (13). Here we show that ESE-1 knockdown in transformed MCF-7 and ZR-75 cells that express endogenous ESE-1, reverses their transformed properties. Moreover, we demonstrate, by ICC, that ESE-1 is localized to the nucleus in MCF-7, T47D and ZR-75 cells, indicating that the ability of ESE-1 to maintain the transformed phenotype requires its role as a nuclear transcription factor. Of note, we have previously demonstrated that GFP-ESE-1 targeted to the nucleus of nontransformed MCF-10A and MCF-12A cells induces apoptosis, whereas transformed T47D and Sk-Br-3 cells tolerate nuclear expression of exogenous ESE-1 without inducing apoptosis, possibly because anti-apoptotic pathways have been up-regulated in these transformed cell. Taken together, these data suggest that ESE-1 initiates transformation in ESE-1-negative mammary epithelial cells via a cytoplasmic- and PAK-1-dependent mechanism, but once fully transformed, these cells require the nuclear transcription properties of ESE-1 to maintain the transformed phenotype.

While dominant-negative ETS approaches, which interfere with multiple ETS factors, have reversed the transformed phenotype in several breast cancer cell lines (NmuMG, MMT and BT20), here we show that the knockdown of a single ETS factor, ESE-1, has the same effect in MCF-7 breast cancer cells. Importantly, we show that ESE-1 is required to maintain the transformed phenotype in MCF-7 breast cancer cells, since shRNA-mediated ablation of endogenous ESE-1 protein resulted in decreased colony formation and anchorage-independent growth. Similar results were obtained with ZR-75 cells, with shESE-1 resulting in decreased colony formation and anchorage-independent growth (data not shown). Furthermore, mechanistic studies, using two separate approaches to measure apoptosis and proliferation, revealed that ESE-1 does not modulate apoptosis, but rather is required for cancer cell proliferation. Finally, we also demonstrate that serum is required to maintain ESE-1 protein production, raising the interesting possibility that reduced ESE-1 protein expression occurring upon serum starvation likely contributes to reduced MCF-7 cell proliferation noted in these conditions. Taken together, this paper contributes novel insights to our understanding of the critical role of ESE-1 in maintaining cell transformation of mammary epithelial cells via regulation of cellular proliferation.

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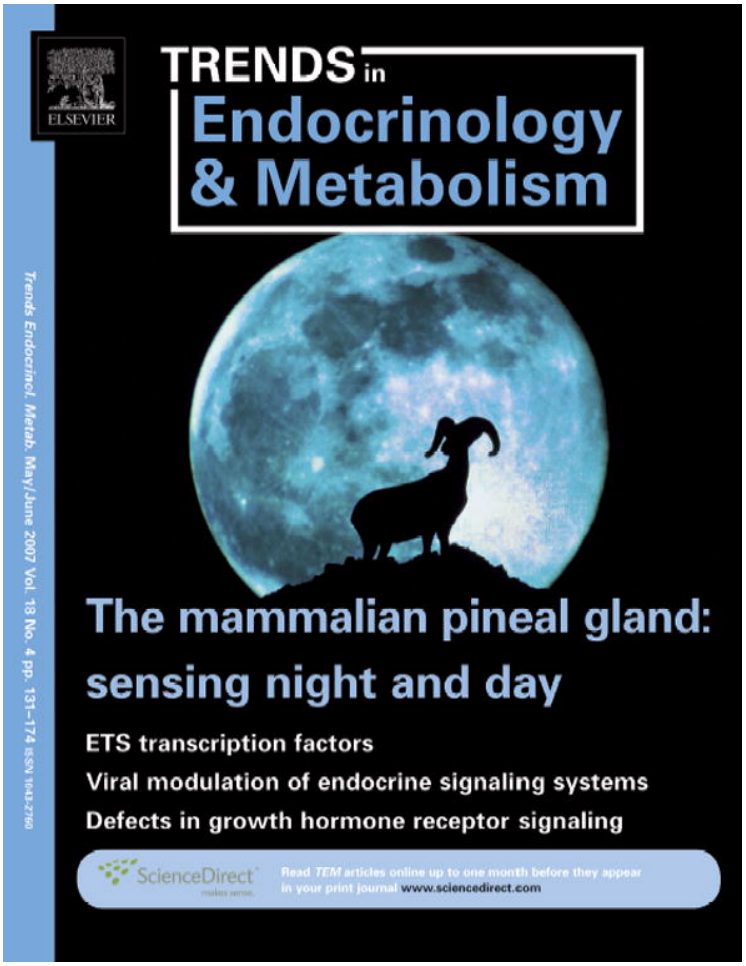
APPENDICES

Enclosed are PDF copies of a published report and a draft of a paper recently submitted to Cancer Research:

1. **Gutierrez-Hartmann A**, Duval DL, Bradford AP. ETS transcription factors in endocrine systems. *Trends Endocrinol Metab*, 18(4): 150-158, 2007.

2. Walker DM, Pozcobutt J, Gonzales MS, Horita H, **Gutierrez-Hartmann A**. ESE-1 is required to maintain the transformed phenotype of MCF-7 and ZR-75 human mammary epithelial cells. *Submitted*.



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ETS transcription factors in endocrine systems

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E26 transformation-specific (ETS) transcription factors have become increasingly recognized as key regulators of differentiation, hormone responses and tumorigenesis in endocrine organs and target tissues. The ETS family is highly diverse, consisting of both transcription activators and repressors that mediate growth factor signaling and regulate gene expression through combinatorial interactions with multiple protein partners on composite DNA elements. ETS proteins have a role in the endocrine system in establishing pituitary-specific gene expression, mammary gland development and cancers of the breast, prostate and reproductive organs.

Introduction: structure and function of ETS proteins

The E26 transformation-specific (ETS) family is restricted to metazoans and thus represents an evolutionarily recent class of transcription factors [1–3]. It is one of the largest transcription factor families, consisting of 27 *ETS* genes in humans, 26 in mice, 10 in *Caenorhabditis elegans* and 9 in *Drosophila* that can be structurally categorized into 11 subfamilies (ETS, ERG, ELG, ELF, ESE, ERF, TEL, PEA3, SPI, TCF and PDEF) [4,5] (Figure 1). The ETS family is defined by the ETS domain, which is a highly conserved DNA-binding domain (DBD) comprising ~85 amino acids (AAs) that is folded into a winged helix-turn-helix DNA-binding motif that binds to a 5′-GGA(A/T)-3′ DNA core motif [2]. All ETS proteins, with the exception of GA-binding protein (GABP)α, bind to DNA as a monomer and are auto-inhibited by virtue of two inhibitory regions that flank the DBD. Disinhibition, resulting in enhancement of ETS DBD activity and of target gene specificity, is achieved by three mechanisms: (i) flanking DNA sequences; (ii) partner–protein interactions; and (iii) kinase-mediated phosphorylation. ETS protein activation of target genes typically involves specific protein–protein interactions and such ETS–protein partner combinations frequently bind to bipartite DNA-binding sites [1–3]. Thus, although target gene selectivity of ETS factors is influenced by specific protein partners, the precise mechanism for achieving DNA-binding specificity, given the high level of redundancy, remains unclear [4,5]. Many ETS subfamilies (ETS, ERG, ELG, ESE, TEL and PDEF) contain the Pointed domain, which serves as a protein–protein interaction motif, several (TEL, ERF and TCF) contain a repressor domain and the majority (ETS, ERG, ELG, PEA3, ESE, SPI and TCF) contain a transcription activation domain (TAD) [1–3]. The activity of certain ETS factors is further regulated by subcellular localization. For example, YAN and TEL (members of the TEL subfamily) NET (also called SAP2, a member of the TCF subfamily) and ERF also contain a nuclear export sequence regulated by mitogen-activated protein kinases (MAPKs) or small ubiquitin-like modifiers (SUMOs), which controls their transcriptional repression activity [2]. By contrast, a cytoplasmic localization is required for ESE1 to transform MCF-12A and MCF-10A human mammary epithelial cell lines [6].

ETS factors are *trans*-acting phosphoproteins that have important roles in epithelial, hematopoietic, neuronal, endothelial and endocrine systems with key roles in cell migration, proliferation, differentiation and oncogenic transformation [1–3,7]. The founding member is encoded by the v-*ets* oncogene in the E26 retrovirus, which causes hematopoietic malignancies in chickens [1–3]. In humans, ETS factors are also associated with several malignancies. For example, chromosomal translocations involving *ETS* genes are found in 95% of human Ewing sarcoma cases and in several human hematopoietic malignancies [1]. In addition, amplification or upregulation of one or more *ETS* genes, including *ETS1*, *ETS2*, *ER81*, *ERM*, *PEA3*, *PDEF* and *ESE1*, is associated with a variety of epithelial cancers (e.g. lung, breast, colon and prostate) [1,2,7]. In mammalian cells, ETS proteins are key nuclear targets of growth factor and *Ras* oncogene signaling pathways, typically acting through the activating protein-1–ETS bipartite Ras response element (RRE) to regulate a repertoire of genes that control cell survival (anti-apoptosis), proliferation and motility [1–3]. Here, we focus on ETS factors in endocrine systems (Figure 2), with particular emphasis on their role in the regulation of pituitary-specific gene expression and tumorigenesis.

ETS factors regulating pituitary function

ETS factors have crucial roles in pituitary gonadotrope and lactotrope biology. Gonadotropes and lactotropes are two of the five hormone-secreting cell types that populate the anterior pituitary gland. Gonadotropes synthesize and secrete the glycoprotein hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH), which regulate gonadal function. Lactotropes produce the protein

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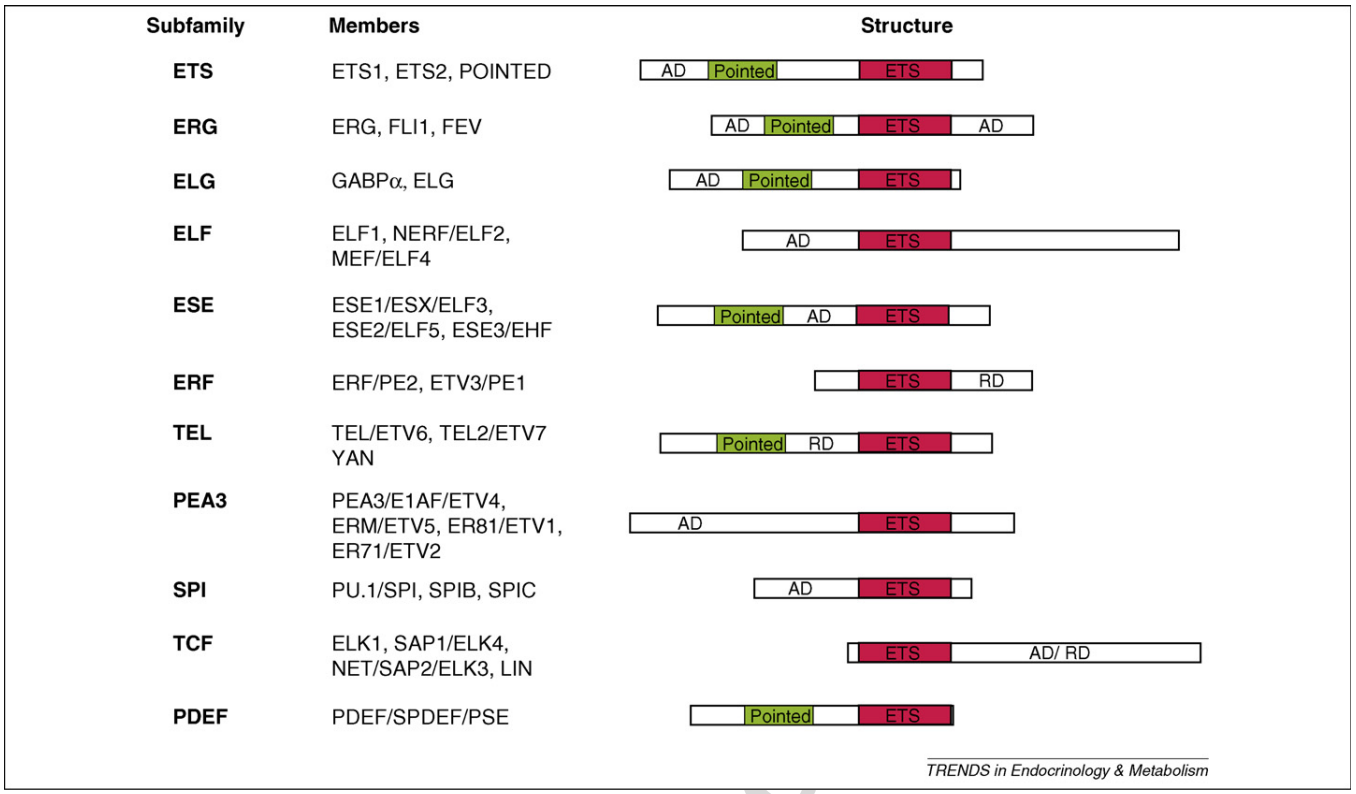

Figure 1. The ETS family of transcription factors. The main functional domains characteristic of members of each ETS sub family are depicted; alternative names for each member are given. Domains: AD, transcriptional activation domain; ETS, DNA binding domain; Pointed, basic helix–loop–helix pointed domain; RD, transcriptional repressor domain. Protein abbreviations: E1AF, E1A enhancer binding protein; EHF, ETS homologous factor; ELF, E74-like factor; ELG, ETS like gene; ER81, ETS related protein 81; ERF, ETS repressor factor; ERG, v-ets avian erythroblastosis virus E26 oncogene related; ERM, ETS related molecule; ESE, Epithelial specific ETS; ETS, v-ets erythroblastosis virus E26 oncogene homolog; ETV, ETS variant gene; FLI1, Friend leukemia virus integration 1; FEV, Fifth Ewing variant; GABP, GA repeat binding protein; LIN, abnormal cell lineage; MEF, myeloid ELF1-like factor; NERF, New ETS-related factor; PEA3, polyomavirus enhancer activator-3; PDEF, prostate derived ETS transcription factor; PSE, prostate epithelium-specific ETS; SAP, Serum response factor accessory protein; SPDEF, SAM pointed domain containing ETS transcription factor; SPI, spleen focus forming virus proviral integration oncogene; TEL, translocation, Ets, leukemia; TCF, Ternary complex factor.

hormone prolactin (PRL), which stimulates breast development and lactation. Hypothalamic gonadotropin-releasing hormone (GnRH) regulates the production and secretion of LH and FSH in pituitary gonatotropes. Stimulation by GnRH leads to MAP kinase activation and ETS factor-mediated induction of the α-subunit gene through a GnRH response element [8]. The α-subunit is a common heterodimeric component of the gonadotrope-specific LH and FSH glycoprotein hormones. Activation of ETS factors in response to GnRH stimulation also induces the expression of the immediate-early gene *Egr-1* through serum response elements [9]. Egr-1, in turn, is a primary activator of the LHβ-subunit gene in response to GnRH stimulation [8]. Thus, ETS factors are crucial mediators of the signaling pathways that regulate the responses of pituitary gonadotropes to hypothalamic GnRH stimulation (Figure 3a).

Pituitary lactotropes exhibit even greater dependence on ETS transcription factors for cellular growth, differentiation and PRL gene expression. Lactotrope cell growth and PRL synthesis and secretion are under tonic inhibitory regulation through hypothalamic-secreted dopamine acting on D2 receptors. The ERF repressor factor is expressed in pituitary lactotrope cell lines and it might be a key contributor to the negative effects of dopamine on PRL gene transcription and lactotrope cell growth [10]. Specifically, dopamine-mediated inhibition of extracellular-signal-regulated kinase (ERK) 1 and ERK2 activity increases ERF repressor action on the rat (r) PRL promoter, whereas growth factor-stimulated ERK activity reverses the repressor function of ERF through ERF phosphorylation [10].

ETS factors also regulate lactotrope differentiation. Pit-1 is a POU homeodomain, pituitary-specific transcription factor that governs the ontogeny of somatotrope, lactotrope and thyrotrope cell types and regulates growth hormone (GH), PRL and thyroid-stimulating hormone β gene transcription [11,12]. In GHFT pituitary precursor cells, which express low levels of Pit-1 but fail to express endogenous PRL or GH, *PRL* gene expression and the lactotrope phenotype is induced only by fibroblast growth factor-2 (FGF-2), and in an ETS-dependent fashion [13]. Specifically, this FGF-2 differentiating response was inhibited by a dominant-negative ETS construct and was augmented by the expression of ETS1. In the GH4 rat somatolactotrope cell line, FGF-2- and FGF-4-mediated induction of rPRL promoter activity is ETS dependent [14], and GH4 cells stably expressing FGF-4 produce tumors that are faster growing and more invasive when injected into Wistar rats [15]. Interestingly, the cooperation of ETS factors with Ikaros, a zinc-finger transcription factor, also regulates expression of the FGF-R4 isoform of the FGF receptors in GH4 somatolactotrope cells [16]. In addition, stable expression of a dominant-negative ETS construct (which encodes only the ETS-2 DBD) in GH4 cells was found to decrease PRL expression but had no effect on

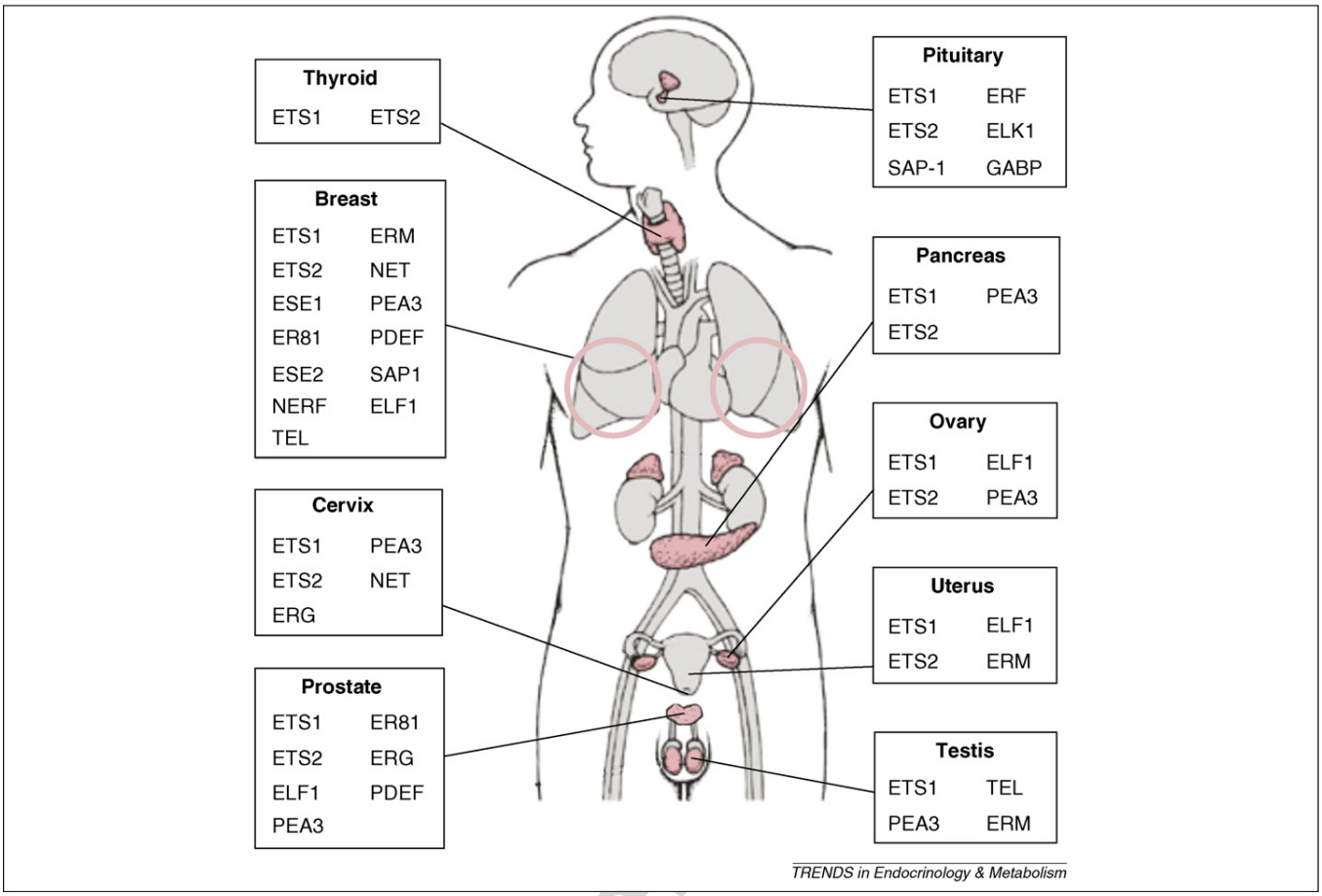

Figure 2. Mammalian ETS factor expression in endocrine organs and target tissues. A list of ETS factors implicated in normal function and/or tumorigenesis is shown for each human tissue. See text for discussion and corresponding references.

GH expression [17]. Furthermore, targeted expression of this same dominant-negative ETS construct to pituitary lactotropes in transgenic mice resulted in diminished lactotrope cell numbers (J. Tentler and A.G.H., unpublished). Taken together, these data reveal that ETS factors might represent a crucial determinant in both dopamine-mediated inhibitory lactotrope regulation and in growth factor-induced ontogeny of pituitary lactotropes.

One of the most characterized systems that best exemplifies the roles of various ETS factors in endocrine gene regulation is the lactotrope-specific *PRL* gene (Figure 3b). The proximal rPRL promoter contains four regions that bind to nuclear proteins derived from GH4 or GH3 rat pituitary somatolactotrope cells, with three of these regions [footprint (FP) I, III and IV] binding to Pit-1 [11,12]. An additional site was identified in promoter mutagenesis studies as the basal transcriptional element (BTE) [11,12]. The BTE site contains an ETS-binding site that is crucial for both basal activity of the PRL promoter and for responses to growth factors, including FGF-2, FGF-4, insulin, insulin-like growth factor, epidermal growth factor (EGF) and thyrotropin-releasing hormone [11,12,18]. Although electrophoretic mobility shift analyses have shown that the BTE site can bind to a variety of ETS factors, including ELK1, SAP1 (also known as ELK4), ETS1 and GABPα (together with its non-DNA-binding partner GABPβ1), BTE DNA-affinity purification of nuclear proteins from GH3 pituitary tumor cells identified the heterodimeric ETS factor GABPα–β1 as the key functional ETS factor binding to the BTE site [18]. Subsequent downregulation of the GABPα and GABPβ1 subunits using small interfering RNA strategies in GH3 cells reduced the expression of endogenous PRL and established the role of GABP as a crucial regulator at the BTE in the PRL promoter [18].

A second crucial ETS regulatory site in the proximal rPRL promoter is a composite RRE, which is constituted by an ETS-binding site adjacent to a Pit-1 binding site, FP IV [3,19]. The *rPRL* RRE binds to ETS1 and Pit-1 and primarily mediates Ras activation of the *rPRL* promoter, but it also contributes to basal activity. Oncogenic V12Ras activates the Raf–MAPK kinase–MAPK signaling cascade, with MAPK directly phosphorylating chicken ETS1 [3]. Site-specific mutation of the ETS1 Thr82 MAPK phosphorylation site to Ala results in the loss of ETS1 enhancement of the Ras response [3]. Mutation of either the ETS1 or Pit-1 site diminishes the Ras response of the *rPRL* promoter in transient transfections of GH4 cells [19]. Similarly, expression of a dominant-negative ETS construct or Pit-1β (an alternatively spliced isoform that functions as a dominant-negative effector in pituitary cells) represses Ras activation of the *rPRL* promoter [3]. Contributing to this ETS–Pit-1 combinatorial code is the physical interaction of the TAD of ETS1 with the Pit-1 homeodomain (AAs 199–291), as demonstrated using

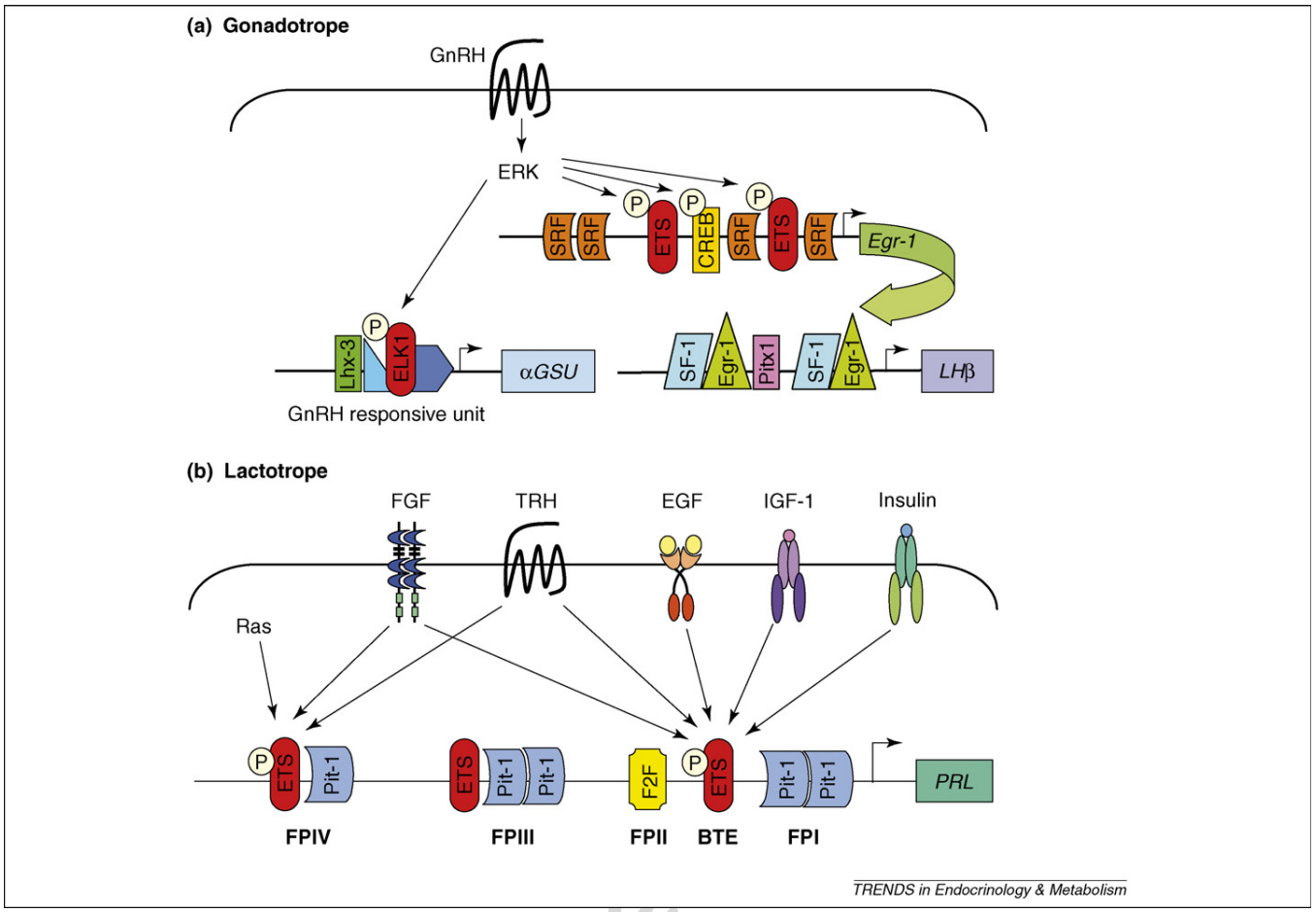

Figure 3. ETS factors as nuclear targets and integrators of signal transduction pathways regulating pituitary gonadotrope and lactotrope gene expression. **(a)** GnRH acting through the ERK pathway directly activates an ETS factor (ELK1) bound to the human α-glycoprotein subunit gene promoter (*αGSU*) within the GnRH responsive unit. Blue triangle, αBP1; hexagon, αBP2. GnRH also stimulates the expression of the human early growth response protein 1 gene (*Egr-1*) through ERK-dependent phosphorylation and activation of ETS factors (ETS) and the cyclic AMP response element binding protein (CREB). Upregulation of the transcription factor Egr-1 subsequently contributes to GnRH stimulation of the human *LHβ*-subunit gene promoter, through a composite response element consisting of binding sites for steroidogenic factor-1 (SF-1), the paired-like homeodomain transcription factor (Pitx1) and Egr-1. **(b)** ETS factors integrate multiple signaling pathways regulating *PRL* gene expression. Stimulation of the proximal –425 rat *PRL* gene promoter (*PRL*), in response to the indicated hormones, growth factors or oncogenic Ras, is mediated through ERK phosphorylation of distinct ETS factors (denoted by the circled P), described in the text, which bind to a composite ETS–Pit-1 binding site (FPIV) and/or an ETS binding site in the BTE. The binding of different ETS proteins to common response elements, and interactions with other transcription factors, provides a mechanism to confer highly specific responses to inductive signals and to coordinate and integrate hormonal and growth factor regulation of *PRL* gene expression. Abbreviations: FGF, fibroblast growth factors; IGF-1, Insulin like growth factor 1; Lhx-3, Lim-homeobox factor 3; SRF, serum response factor; TRH, thyroid-releasing hormone.

NMR approaches [20] (Figure 4). In addition, Pit-1 phosphorylation at Thr220, within the homeodomain, regulates the binding of Pit-1 to both ETS1 and the monomeric Pit-1 binding site within the RRE [20]. However, Pit-1 is also a unique cell-specific target for Ras signaling, and mutation analyses of Pit-1 have localized Ras responsiveness to AAs 60–80, at the C-terminus of the TAD [21]. This Pit-1 TAD region contributes to the Ras response through the p160 steroid receptor coactivator-1 [21]. Thus, the binding of Pit-1 and ETS1 to the composite RRE in the proximal *rPRL* promoter seems to generate a unique binding platform for Ras-stimulated coactivator complexes.

ETS proteins in mammary gland development and breast cancer

During embryogenesis, ETS1, PEA3, ERM, ER81 and ESE1 are expressed in the mammary gland, and the PEA3 subfamily members PEA3, ERM and ER81 have been shown to be expressed throughout mammary gland development [7,22–24]. *In situ* hybridization approaches have revealed that ERM and PEA3 are expressed in epithelial cells of the developing terminal end buds, suggesting that they have a role in branching morphogenesis [24]. *PEA3* knockout mice show an increased number of proliferating cells and increased terminal end buds, implying that PEA3 functions as a repressor of mammary epithelial cell growth [24].

In postnatal mammary glands, ETS factors have been shown to have key roles in pregnancy-induced, PRL-mediated mammary gland lobuloalveolar development and milk production and in breast tumorigenesis. In the early phase of pregnancy, a proliferative phase of mammary alveolar morphogenesis mediated by PRL and signal transducer and activation of transcription (STAT)5 is initiated, and the PRL–STAT5 pathway is responsible for the development of lobuloalveoli and the induction of lactation. Studies using *elf5* and *prlr* gene knockout mice and rescue by targeted re-expression of *elf5* in *prlr* nullizygous mammary epithelium revealed that the ETS factor Elf5 (also known as Ese-2) is the crucial downstream

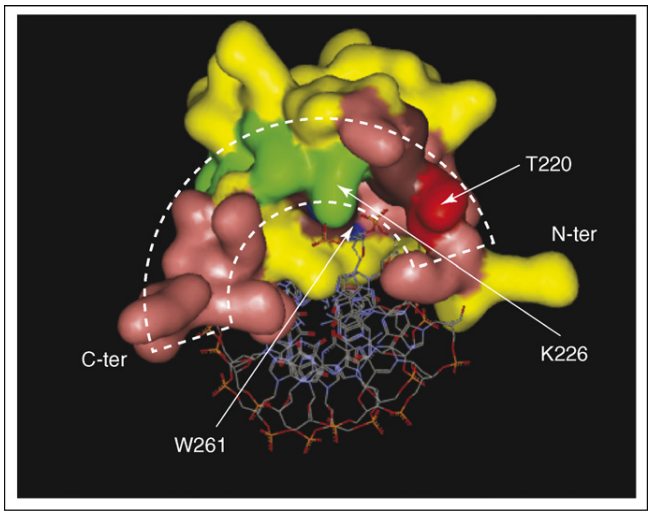

Figure 4. Chemical shift perturbations of the Pit-1 homeodomain induced by the binding of ETS1. A surface density representation of the Pit-1 homeodomain bound to DNA based on the crystal structure of the Pit-1 homeodomain. NMR shift perturbations of residues in the Pit-1 homeodomain (amino acids z199–291), in response to the binding of the Region III TAD (amino acids 190–257) of ETS1 [20], are indicated by color coding: red > purple > pink > green. Yellow residues are unaffected. The ETS interaction face on the Pit-1 homeodomain is indicated by a dashed white line. Amino acids highlighted in the ETS1 interaction face seem to affect the ETS1–Pit-1 interaction. W261 (blue) is in the hydrophobic DNA binding pocket, T220 (red) is a phosphorylation site and K226 (green) is a potential acetylation site. Adapted from Ref. [20]; copyright 2002 National Academy of Sciences USA.

effector of the PRL–STAT5 signaling pathway [25,26]. Thus, Elf5 (Ese-2) is necessary and sufficient for lactation-competent mammary gland development during pregnancy.

Several different lines of evidence suggest that ETS factors have a particularly relevant role in breast cancer. Although many ETS factors have been shown to be overexpressed in breast cancer, most of these studies have focused on mRNA rather than protein expression, and few have examined compartment-specific expression [7,22–24]. A recent comprehensive quantitative PCR and subtractive tissue analyses of 25 different ETS factors in normal and malignant murine mammary gland tissues and cell lines revealed that multiple ETS factors are expressed in lymph, stromal and epithelial mammary compartments [4]. For example, ELK4, ELF1 and ETS2 are the most abundant ETS factors expressed in the normal mammary gland, whereas PDEF, PEA3, ESE1, ESE2, ESE3, TEL (also known as ETV6),and NERF (also known as ELF2) mRNAs displayed significantly elevated expression in the epithelial cell compartment of mammary tumors [4]. Important data revealing a direct role of ETS proteins in mammary tumorigenesis have been reported, showing that a dominant-negative ETS2 DBD can block the anchorage-independent growth and cellular invasiveness of the NmuMG, MMT and BT20 breast cancer cell lines [27,28]. However, some of the most convincing reports stem from transgenic mice studies, showing that compound *ets2* knockout MMTV–polyoma middle T (PyMT) mice, in which one *ets2* allele has been knocked out, are more resistant to PyMT-induced breast tumorigenesis than are their wild-type counterparts [29]. Similarly, the generation of female mice expressing a homozygous targeted *ets2* gene, *ets2(A72/A72)*, which precludes ERK phosphorylation of Thr72, blocked mammary tumors caused by transgenic-targeted oncogenes and seemed to do this exclusively through a stromal location [30,31]. Additionally, using immunohistochemical and western blotting approaches, several studies have revealed that specific ETS proteins, including members of the ETS, PEA3 and ESE subfamilies, are upregulated in breast cancer tumors and cell lines [7,22–24]. Although increased PEA3 and PDEF (also called PSE) mRNAs have been associated with breast cancer [7,22–24], their precise role in mammary tumorigenesis has been controversial. Studies testing their ability to transform mammary epithelial cells reveal that PEA3 and PDEF actually inhibit breast cancer cell growth [32–35]. Thus, although most reports implicate ETS factors in breast cancer, only the ETS factor ESE1 has been shown to confer an epithelial-to-mesenchymal transition phenotype and actually to transform human mammary epithelial cells [6,36–38].

ESE1 (also known as ESX or ELF3) is an epithelial-specific ETS protein that is particularly relevant to breast cancer because the *ESE1* gene maps to human chromosome 1q32.1, in a region that is overrepresented in 50% of early breast cancers [7]. ESE1 mRNA and protein is overexpressed in human breast ductal carcinoma *in situ*, an early cancer stage that also overexpresses HER2 (also known as neu) [7]. Furthermore, a positive feedback loop between the *HER2* proto-oncogene and ESE1 seems to exist [7]. ESE1 contains several unique features among ETS factors. For example, its mRNA expression is restricted to the mammary ductal epithelia and terminal ductal–lobular units, which are the most terminally differentiated, epithelial-derived cells [7]. Unique to ESE1 among ETS proteins are a 40-AA serine- and aspartic rich (SAR) motif and an HMG-like AT-hook domain, in addition to the Pointed, TAD and ETS DBD domains found in most ETS factors [7] (Figure 5a). Recent work revealed that ESE1 is required for the transformed phenotype of $HER2^{+}$ T47D breast cancer cells [36], that it transforms the immortalized but nontransformed MCF-12A and MCF-10A human mammary epithelial cells and that it does so through an autonomously functioning, unique 40-AA SAR domain acting through a novel cytoplasmic mechanism [6,37] (Figure 5b). A subsequent study screening ~250 cDNAs implicated in breast cancer independently identified ESE1 as being able to confer a motile phenotype upon MCF-10A cells, resulting in disorganized organoids in 3D culture [38], as previously reported [37]. The discovery that ESE1 functions in the cytoplasm to transform mammary epithelial cells established novel paradigms for ETS factor function and mechanisms by which transcription factors induce cell transformation.

ETS factors in the prostate

The expression of several ETS factors in the normal and/or cancerous prostate has been reported, including ETS1, ETS2, ELF1, ESE2 (also called ELF5), ER81, ERG, PDEF and PEA3, with ETS fusions having become one of the most common genetic markers of prostate cancer [1,39]. The ETS factor PDEF/PSE was originally isolated as a transcriptional regulator of the prostate-specific antigen gene

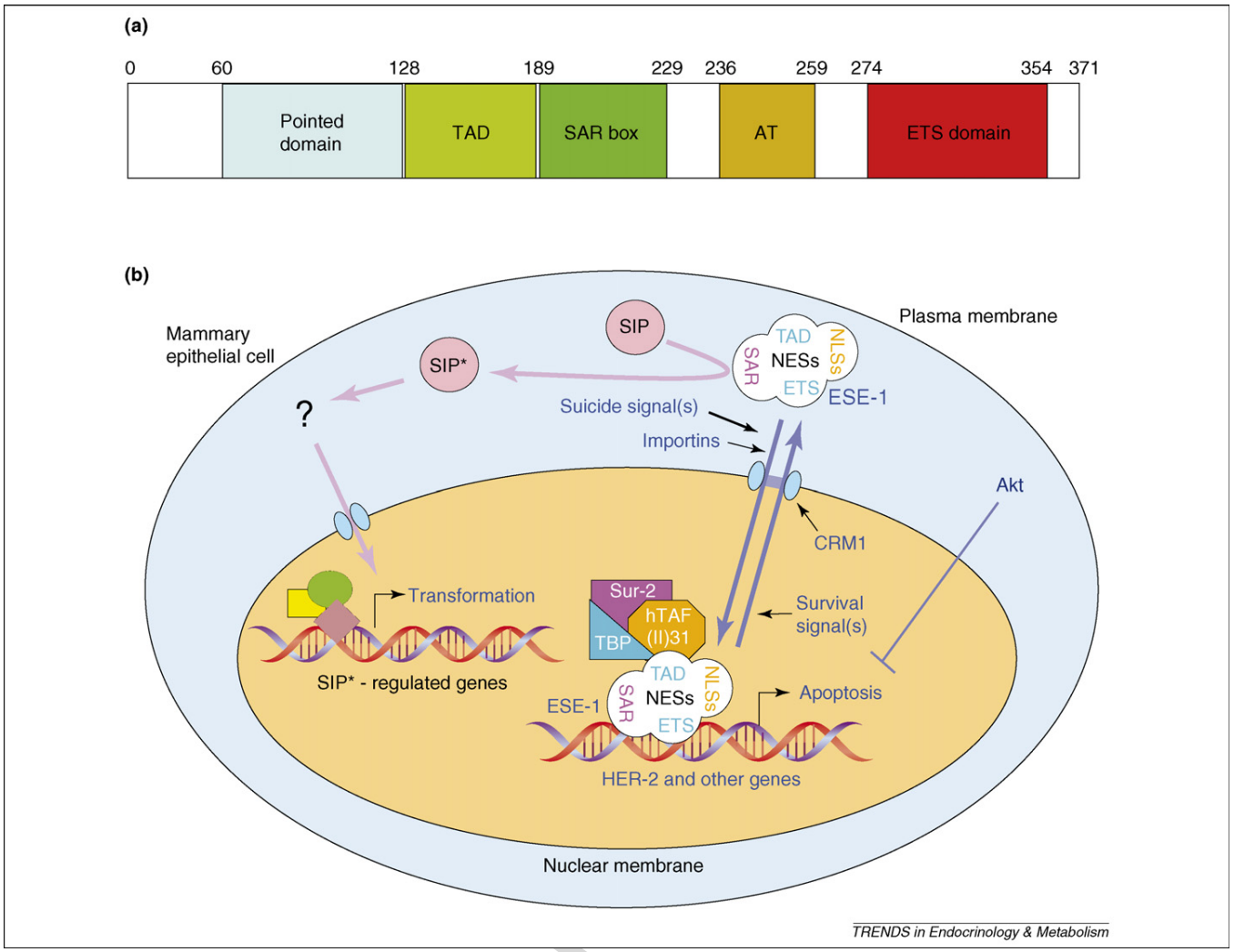

Figure 5. **(a)** Domain organization of ESE1. Shown is the 371 amino acid ESE1 protein, with the Pointed, TAD, SAR domain, AT-hook domain, and ETS DBD. Endpoints (residue numbers) are shown above each domain. **(b)** Model of ESE1 function in mammary gland biology. ESE1 is shown in the nucleus as binding to a target gene and activating an apoptotic pathway in normal epithelium, with nuclear localization governed by suicide signals. By contrast, survival signals are postulated to induce cytoplasmic localization of ESE1, through a Chromosomal region maintenance 1 (CRM1)-dependent mechanism, which is required for ESE1 to transform human mammary epithelial cells. The SAR domain is proposed to interact with SAR-interacting protein (SIP), converting it to the active form, SIP*, which then activates an unknown signal transduction pathway (shown as?), resulting in specific gene(s) transcription that establishes the transformed phenotype. Abbreviation: NES, nuclear export sequence; NLS, nuclear localization sequence; TAF, transcription activating factor; TBP, TATA binding protein.

[7]. Subsequently, PDEF/PSE mRNA was found to be increased in breast and prostate cancer, yet PDEF/PSE protein expression was found to be decreased as the prostate malignancy grade progressed [7]. These data have been interpreted to indicate that PDEF might actually function as a tumor suppressor and that a translation control mechanism seems to regulate PDEF protein expression in both prostate and breast cancers [7,32,35].

The first clinically relevant candidates for dominant oncogenes in prostate cancer are ETS fusion genes resulting from chromosomal translocation of the 5′ untranslated region of a prostate-specific, androgen-responsive, transmembrane serine protease gene (*TMPRSS2*) to *ERG*, *ER81* (also known as *ETV1*) and *PEA3* (also known as *ETV4*) ETS transcription factor genes [39]. Deletion of genomic DNA between *TMPRSS2* (21q21.3) and *ERG* (21q21.2) results in the TMPRSS2:ERG fusion protein in ~50% of prostate cancers and chromosomal rearrangements between *TMPRSS2* (21q21.3) and *ER81* (7p21.2) or *PEA3* (17q21) occur in another ~30%, indicating that *TMPRSS2–ETS* gene fusions might be the most common genetic abnormality defined to date in human malignancies [39]. These fusions result in androgen-mediated, robust induction of these various ETS factors, which are then thought to activate a repertoire of ETS-responsive genes, leading to prostate cell transformation [39].

ETS factors in reproductive tissues

The ETS family of transcription factors has crucial roles in the regulation of reproduction and embryogenesis. In the male, ERM is required in testicular Sertoli cells for spermatogonial stem cell self-renewal and transcriptional regulation of the stem cell niche [40]. Testicular germ cell tumors show allelic imbalance in the chromosomal region encoding TEL [41], and increased expression of ETS1 was associated with metastasis and angiogenesis [42]. PEA3 expression was increased in testicular seminomas, correlating with enhanced matrix metalloprotease (MMP) 2 levels [43].

In the female, uterine expression of ETS1 and the PEA3 subfamily have been implicated in endometrial angiogenesis and implantation [44,45]. ETS1 is also a crucial regulator of the uterine decidual PRL promoter and is dramatically upregulated during decidualization of the endometrial stroma [46]. In early postimplantation development, ETS2 expression is restricted to placental trophoblasts and is essential for placental function. Deletion of the DNA-binding domain of ETS2 in transgenic mice resulted in embryonic death before day 8.5, owing to defects in extra-embryonic tissue, including deficient MMP9 activity and failure of ectoplacental cone proliferation [47]. Subsequent analysis indicated that ETS2 is necessary for the expression of extra-embryonic ectoderm (EXE) markers and anteroposterior patterning mediated by EXE-derived signals [48]. Deletion of a second trophoblast-specific ETS-related gene, *elf-5*, also results in conceptuses lacking EXE [49].

Several ETS factors have been investigated as prognostic markers in gynecological tumors. In cervical cancer, overexpression of ETS1, ERM (also called ETV5), ERG or ETS2 was associated with higher grade, metastasis and poor prognosis and correlated with elevated levels of MMP-1 and increased angiogenesis [50,51]. A polymorphism of the MMP-1 promoter, which generates an additional ETS-binding site, was also linked to advanced clinical stage and reduced survival in cervical and endometrial cancer [52,53]. Loss of the ETS repressor protein NET (also called SAP2) resulted in enhanced c-Fos expression in cervical cancer cells, a key event in transformation [54]. By contrast, expression of PEA3 suppressed the invasiveness of cervical carcinoma cells [55].

In endometrial tumors, increased expression of ETS1 and ELF1 is associated with advanced surgical stage, enhanced invasion and angiogenesis, and poor prognosis linked to elevated levels of MMPs [56,57]. ERM was also upregulated in endometrial carcinoma in tumor stages associated with myometrial invasion [58]. Accordingly, treatment of progesterone receptor B-expressing Ishikawa endometrial cancer cells with progesterone resulted in inhibition of cell growth and invasion, concomitant with decreased levels of ETS1 and MMPs [59], whereas estrogen-stimulated growth and invasion was associated with increased ETS1 and MMP expression [60]. Overall, evidence indicates that ETS factors, by virtue of their transcriptional regulation of MMPs and angiogenic genes, are crucial mediators of angiogenesis, invasion and metastasis in the development and progression of gynecological malignancies.

Increases in ETS1 and ELF1 are also linked to the malignant potential of ovarian cancer, being associated with higher grade and poor prognosis [61,62]. Over expression of ETS1 in ovarian cancer cells also conferred resistance to chemotherapy [63]. Similarly to cervical cancer, expression of ETS1 and PEA3 correlated with elevated MMPs 1, 2 and 9 and increased angiogenesis, suggesting a central role for these ETS factors in the progression of ovarian carcinoma [64,65]. However, consistent with its inhibition of cervical cancer cell invasion, PEA3 is also reported to downregulate *Her2* gene expression and reverse the transformed phenotype of ovarian cancer cells *in vitro* [66].

ETS factors in thyroid and pancreatic malignancies

Evidence suggests a role for ETS1 and ETS2 in the pathogenesis of thyroid cancer. Expression of ETS1 is increased in papillary and follicular thyroid carcinomas relative to benign nodules or normal tissue [67]. ETS1 and ETS2 expression and transcriptional activity were also increased in thyroid cancer cells. A dominant-negative ETS construct suppressed anchorage-independent growth and induced apoptosis in thyroid carcinoma but not in normal thyroid cell lines, implying a requirement for ETS1 and ETS2 to maintain the transformed phenotype [68]. In pancreatic adenocarcinoma, particularly in late-stage invasive tumors, elevated ETS2 protein was detected and PEA3 was shown to activate the mucin 4 promoter, an epithelial marker of pancreatic ductal carcinoma not present in normal tissue, suggesting that ETS factors might also be important in pancreatic cancer [69,70]. Finally, ETS1 has been implicated in ectopic expression of the endocrine ligand parathyroid hormone-related peptide, which is associated with bone metastases and hypercalcemia in several cancers, including breast cancer [71].

Conclusion

In summary, ETS factors have been shown to have crucial roles in development, differentiation and tumorigenesis in several endocrine systems. Studies in the pituitary, mammary and prostate glands have provided compelling insights into the functional role of specific ETS factors in these endocrine tissues. Emerging data in many other endocrine organs suggest a broader role for ETS transcription factors in endocrine pathophysiology. Future studies, targeting select endocrine tissues, by generating tissue-specific knockouts of individual ETS genes or expressing a dominant-negative ETS transgene, will provide an even greater understanding of the role of ETS factors in endocrine biology.

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ESE-1 is required to maintain the transformed phenotype of MCF-7 and ZR-75 human mammary epithelial cells

Running title: ESE-1 maintains mammary cell transformation

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ABSTRACT

ETS factors comprise a large transcription factor family known to play a significant role in cellular development, differentiation, and transformation. Emerging evidence reveals that increased mRNA expression of the human Ets factor-1, ESE-1, is associated with breast cancer. Stable expression of ESE-1 transforms MCF-12A immortalized human mammary epithelial cells. However, little is known about ESE-1 protein expression and its role in maintaining the transformed phenotype in human breast cancer cell lines. Here, we used an anti-ESE-1 mouse monoclonal antibody in Western blot and immunofluorescent cell analyses to show that ESE-1 is expressed as a nuclear protein in MCF-7, T47D and ZR-75 transformed, tumorigenic mammary epithelial cell lines, and that it is not expressed in transformed MDA-MB-231 and nontransformed MCF-10A and MCF-12A cells. In addition, specific knockdown of endogenous ESE-1 in the human breast carcinoma ZR-75 and MCF-7 cell lines decreased colony formation and anchorage independent growth. Mechanistically, ESE-1 knockdown decreased cellular proliferation, but had no effect on apoptosis. Finally, serum withdrawal resulted in a time-dependent, ~90% reduction of ESE-1 protein production in MCF-7 cells. These results establish that ESE-1 plays a key role in maintenaning the transformed phenotype in breast cancer, thus providing a novel single-point target for breast cancer therapy.

INTRODUCTION

The ETS transcription factor family is composed of 27 members in humans, and ETS proteins appear to have important roles in cellular proliferation, differentiation and transformation (1-3). This large family of transcription factors is characterized by a conserved winged helix-turn-helix DNA binding domain (DBD), the ETS domain, which mediates binding to target DNA sequences (3). ETS proteins function as transcriptional activators or repressors, and are regulated by protein-protein interactions and mitogen-activated protein kinase (MAPK) phosphorylation (1-3). In particular, activation of the Ras proto-oncogene has been shown to mediate phosphorylation of several ETS factors (3, 4). This is significant because the Ras pathway is critical in regulating cell cycle and proliferation (3, 4). This, in conjunction with *ETS* chromosomal translocations and over-expression, suggests that this family has a major role in oncogenesis (1, 5, 6).

Epithelial specific ETS factor-1, ESE-1 (also known as ESX, Jen and ERT, and Elf3 in mice) is a ~42 kDa protein, and it is the defining member of the epithelial-restricted, ESE subfamily of ETS transcription factors (7-10). The ESE-1 cDNA was first isolated while screening cDNA from human keratinocytes and pancreatic cancer tissue for novel transcripts containing the ETS domain (7, 9). Since ESE-1's initial isolation, its mRNA expression has been documented in several human and rodent epithelial tissues, including placenta, lung, kidney, prostate, intestine, breast, skin, retina and other epithelia (7-10). During mouse embryo development, Elf3 mRNA expression levels increase progressively, from embryonic day 7 to day 17, and in post-embryonic mammary gland development, Elf3 is found in virgin, pregnant and involuting mammary glands (11). Furthermore, over-expression of ESE-1 mRNA has been demonstrated in nearly 50% of early human breast tumors, and its expression is increased significantly in ductal carcinoma *in situ* (DCIS) (10, 12, 13). This increased ESE-1 mRNA expression

correlates with increased expression of the *Her2/neu* proto-oncogene in breast cancers, and activation of the Her2/Neu receptor induces *ESE-1* gene transcription (12-14). Therefore, a positive-feedback-loop is predicted based on increased ESE-1 expression in response to Her2/neu receptor activation and ESE-1 protein then binding and activating the *Her2/neu* promoter (14-16).

An increasingly large body of evidence reveals that ETS factors are particularly important in breast cancer (1, 5). A thorough mRNA expression analysis of 25 ETS factor members in normal and cancerous mouse mammary glands showed that expression of Pdef, Pea3, Elf3/ESE-1, Elf5/ESE-2, Ehf/ESE-3, ETV6/TEL, and Elf2/NERF mRNAs was elevated in the epithelial cell compartment of mammary tumors (17). Moreover, expression of a dominant-negative ETS, to overcome the redundancy of ETS factor expression, reversed the transformed phenotype in NmuMG, MMT and BT20 breast cancer cell lines (18, 19). To determine whether a single ETS factor could impose the transformed phenotype, we stably expressed HA-ESE-1 or GFP-ESE-1 fusions in the ESE-1-negative, non-transformed MCF-10A and MCF-12A human mammary cell lines, and demonstrated that ectopic ESE-1 increased cellular proliferation, migration, invasion and colony number in soft agar (20, 21). Moreover, ESE-1 was identified as a factor enhancing cell migration and altered morphogenesis in 3D assays in a separate and unbiased analysis of a collection of 1000 cDNAs relevant to breast cancer, in which each of 1000 cDNAs were ectopically expressed in MCF-10A cells (22). Finally, we have reported that ESE-1 initiates transformation of MCF-12A mammary epithelial cells via an autonomously functioning, unique 40-amino acid serine and aspartic rich (SAR) domain acting via a novel cytoplasmic mechanism (20). While dominant-negative and gain-of-function experiments demonstrate that ETS factors, in

particular ESE-1, mediate the transformed state in breast cancer, no study to date has demonstrated that ESE-1 alone is required to maintain the transformed phenotype.

To investigate the potential role of ESE-1 in maintaining a malignant phenotype, we used shRNA targeting ESE-1 and a highly-specific ESE-1 mouse monoclonal antibody (Walker et al, in preparation), in order to characterize and monitor ESE-1 protein expression in several nontransformed and transformed mammary epithelial cell lines. Here we show that shRNA targeting ESE-1 specifically ablates endogenous ESE-1 expression in ZR-75 and MCF-7 cells, leading to an inhibition of clonogenicity and anchorage independent colony growth in these cells. Moreover, we show that ESE-1 knockdown did not induce apoptosis, but rather diminished MCF-7 cellular proliferation. Finally, serum starvation significantly reduced ESE-1 protein expression in MCF-7 cells. These results establish that ESE-1 plays a critical role in maintaining the transformed state and that it does so by controlling cell proliferation. Thus, ESE-1 provides a potential single-point target for future breast cancer therapy.

RESULTS

MCF-7, T47D and ZR-75 human breast cancer cells express ESE-1 protein, whereas transformed MDA-231 and nontransformed MCF-10A and MCF-12A cells do not

In order to characterize and monitor ESE-1 protein expression in basal and shRNA knockdown conditions in various human mammary epithelial cell lines, we generated several highly specific mouse monoclonal antibodies against an ESE-1 peptide spanning amino acids 128-259. This region includes the transcription activation domain (TAD), SAR, and AT-hook domains. Details describing the generation and characterization of these antibodies will be presented elsewhere (Walker DM, in preparation), but in this

report we limited our studies to the use of one of these, anti-ESE-1 mAB405. Western blot analysis of whole cell extracts probing for endogenous ESE-1 was performed on a series of human transformed and nontransformed mammary epithelial cell lines and compared with qRT-PCR. As shown in Fig. 1A, ESE-1 protein was not detected by Western blot analysis in the nontransformed MCF-10A and MCF-12A mammary epithelial cells lines. In contrast, ESE-1 protein was detected in the tumorigenic MCF-7, T47D and ZR-75 cell lines, with the levels in MCF-7 and T47D being equivalent and greater than that expressed in ZR-75. Noteworthy, ESE-1 protein was not detectable in the highly metastatic MDA-MB-231 cells (Fig. 1A). In order to obtain a direct comparison of ESE-1 protein and mRNA levels in this same panel of mammary epithelial cell lines, we next performed a quantitative RT-PCR analysis. This qRT-PCR study revealed that MCF-10A and MCF-12A nontransformed cells do not express any detectable ESE-1 mRNA, whereas the transformed MCF-7, T47D, ZR-75 and MDA-MB-231 cells all express ESE-1 mRNA, but to varying degrees (Fig. 1B). The MCF-10A, MCF-12A and MCF-7 mRNA and protein data do correlate with each other (Fig. 1). However, for each given amount of ESE-1 protein expressed, the T47D mRNA level is lower than expected, whereas the ZR-75 and MDA-MB-231 mRNA levels are higher than expected (Fig. 1). To further confirm ESE-1 protein expression levels and its subcellular localization, we performed indirect immunofluorescence cytochemistry (ICC) studies, with cell nuclei counterstained with DAPI to define each cell (Fig. 2). These data revealed that MCF-10A, MCF-12A and MDA-MB-231 cells fail to express any ESE-1 protein detectable by this ICC method, whereas endogenous ESE-1 protein was detected in transformed MCF-7, T47D and ZR-75, and in each case ESE-1 was localized to the nucleus (Fig. 2). However, the ICC and Western blot data did not strictly correlate, since T47D cells displayed the strongest ICC signal, yet in the Western blot, the T47D signal was equivalent to the MCF-7 lane, but more than the ZR-75 lane (Figs. 1 and 2). As a

negative control, primary anti-ESE-1 monoclonal antibody was omitted for each cell line, and this study revealed that the ICC signal is dependent on the primary anti-ESE-1 antibody (Fig. 2, lower panel). In general, the ICC data further confirmed the Western blot data and revealed ESE-1 protein to be primarily localized in the nucleus.

shRNA targeting of ESE-1 knocks down endogenous ESE-1 protein expression

ZR-75 and MCF-7 are estrogen receptor (ER)-positive human breast cancer cell lines, with the latter being a classic model system used to study estrogen-dependent tumorigenesis. The detection of endogenous ESE-1 in ZR-75 and MCF-7 cells makes these ideal cell lines to study the role of ESE-1 in maintaining tumorigenesis, and whether ESE-1 contributes to cellular survival, apoptosis and/or proliferation. To address these points, we used an shRNA approach to knockdown endogenous ESE-1 in ZR-75 and MCF-7 cells. In initial optimization studies of several shESE-1 constructs, we identified an shESE-1 construct (shESE-1), which targeted the ETS DBD of ESE-1, that optimally knocked down endogenous ESE-1 (data not shown). Using shESE-1, we first sought to establish the time course of ESE-1 knockdown by transiently transfecting ZR-75 cells with shESE-1 and preparing whole cell lysates 2, 3, 4 and 5 days post-transfection (Fig. 3A). As a control, we transfected cells with an shRNA empty vector control (vector) and prepared whole cell lysates 2 days post-transfection (Fig. 3A). The whole cell lysates were then probed for ESE-1 and tubulin by Western blot analysis (Fig. 3A). These results show that compared to vector control, essentially a complete knockdown of ESE-1 occurs by 2 days, and a significant reduction of ESE-1 persists up to 5 days post-transfection of shESE-1 (Fig. 3A). The tubulin control shows that a nearly equivalent amount of protein was loaded in each lane, indicating that differences in protein loading fail to explain the significant reduction in ESE-1 detected. A similar time

course of shESE-1 knockdown was performed in MCF-7 cells and this study showed the same complete reduction of ESE-1 by 2 days, but in MCF-7 cells, this strong level of inhibition persisted up to 5 days (data not shown). Next, we sought to establish the specificity of ESE-1 knockdown. As noted above, this shESE-1 targeted the ETS DBD, which is conserved amongst ETS proteins. Computational analysis of the shESE-1 target sequence revealed it to be unique to ESE-1, with our target sequence showing minimal similarity only to ETS-1/ETS-2 (with only 4 of 19 nt being identical for each). As a negative control (shCtr), we used an shRNA construct that also targeted the ESE-1 ETS DBD, but which in optimization studies failed to inhibit ESE-1 expression. We transiently transfected MCF-7 cells with shCtr and shESE-1 shRNA vectors, prepared whole cell lysates 2 days post-transfection, and probed for ESE-1, Ets-1/Ets-2 and tubulin by Western blot analysis (Fig. 3B). This study reveals that the shCtr failed to inhibit endogenous ESE-1, while the shESE-1 vector resulted in a robust knockdown of ESE-1 in MCF-7 cells. We quantitated this inhibition by normalizing ESE-1 expression to tubulin and found that ESE-1 expression is reduced ~4-fold in the shESE-1 cells compared to shCtr (Fig. 3C). Finally, to determine the specificity of shESE-1 knockdown, we performed Western blot analysis for both ETS-1 and ETS-2, using an antibody that recognizes both ETS factors. As shown in Fig. 3B, neither the shCtr nor shESE-1 affected the levels of ETS-1 plus ETS-2, affirming shESE-1's specificity to knockdown endogenous ESE-1, and that the shCtr failed to inhibit ESE-1, ETS-1 and ETS-2.

Knockdown of ESE-1 reduces the colony-forming ability of ZR-75 and MCF-7 cells

In order to determine if ESE-1 was necessary for colony formation, ZR-75 and MCF-7 cells were each co-transfected with pEGFP-C3, to confer G418 resistance, and shCtr or

shESE-1. Transfected cells were selected with G-418 for 14 days, resultant colonies were stained with Crystal violet, and counted by direct visualization (Fig. 4A and B). The ZR-75 and the MCF-7 cells transfected with shCtr yielded ~43 and ~27 colonies per plate, respectively, with ZR-75 colonies being larger than the MCF-7 colonies (Fig. 4A). Quantitation of triplicate colony formation assays indicated that only ~three ZR-75 colonies formed in the presence of shESE-1, resulting in a 13-fold reduction in ZR-75 colony formation and only ~six MCF-7 colonies formed in the presence of shESE-1, resulting in a 5.5-fold reduction in MCF-7 colony formation (Fig. 4B). Of note, we show a 14-day selection with G418, since several attempts to generate stable ESE-1 knockdown cell lines resulted in very few, small colonies that failed to grow, thus making clonal expansion unsuccessful. Importantly, the similar inhibitory effect of ESE-1 knockdown on colony formation in two distinct breast cancer cell lines supports the critical role of ESE-1 in the growth of transformed mammary cells.

Knockdown of ESE-1 inhibits anchorage independent growth of MCF-7 cells

Soft agar growth assays are classic in vitro experiments to test for anchorage independent growth. To further investigate the functional role of ESE-1 in the tumorigenic phenotype, we performed similar shRNA knockdown studies of ESE-1 and performed soft agar experiments using MCF-7 cells. We chose to focus on MCF-7 cells for these soft agar experiments, because MCF-7 cells yielded larger colonies than ZR-75 cells in soft agar, allowing for easier quantitation of shESE-1 effects. Moreover, previous optimization studies revealed that transient transfection of MCF-7 cells with shESE-1 resulted in a more effective and prolonged knockdown of endogenous ESE-1 (data not shown). This more prolonged knockdown after MCF-7 transient transfection is important, since the soft agar assay is over 14 days and we could not generate stable

shESE-1 knockdown cells to then plate in soft agar. Because in the transient transfection approach not all cells are transfected and ESE-1 expression is likely to re-appear at later time points, compared to G418 selection methods, the resulting colony number in the shESE-1 knockdown cells presented here is likely an overestimate. Thus, we transiently transfected MCF-7 cells with shCtr or shESE-1 vector DNAs, plated the cells in soft agar and after 14 days counted the colonies growing in an anchorage independent manner (Fig. 4C and D). Shown in Fig. 4C is a representative digital image of shCtr- and shESE-1-transfected MCF-7 cells growing as colonies in soft agar. The shCtr image shows many more colonies, compared to the shESE-1 image. Furthermore, in colonies meeting the threshold limit, there were no marked differences in colony size between both groups (data not shown). MCF-7 cells transfected with shCtr generated ~800 colonies, whereas shESE-1-transfected cells generated ~350 colonies, a 56% reduction in colony formation (Fig. 4D, plated in sextuplicate). A separate study, completed in triplicate, showed that shESE-1 mediated a 64% reduction in MCF-7 soft agar colony number, with MCF-7 cells transiently transfected with shCTR- and shESE-1 yielding 575 and 209 soft agar colonies, respectively (data not shown). Cloning efficiency was determined by dividing the number of cells seeded by the number of colonies formed times 100. Control cells yielded a cloning efficiency of 1.6%, while shESE-1 cells had a cloning efficiency of 0.7%. Thus, taken together, these functional assays indicate that ESE-1 is required to maintain the tumorigenic phenotype of MCF-7 cells.

Reversion of the Transformed Phenotype is not due to Apoptosis

Having demonstrated a reduction in colony formation and anchorage independent growth, we next sought to address the mechanism responsible for the reversion of the

malignant phenotype. We first tested whether knockdown of ESE-1 in MCF-7 cells resulted in apoptosis. In order to address this point, MCF-7 cells were transiently transfected with empty vector, shCtr or shESE-1, and harvested at 48 and 72 hours. We tested for apoptosis by using two separate assays, DNA laddering (Fig. 5A) and caspase 3/7 analyses (Fig. 5B and C). The DNA laddering assay showed no DNA smearing at the 48-hour time point for vector control, indicating that the transfection method alone did not induce apoptosis (Fig. 5A, lane 1). Similarly, there was no DNA smearing at the 48-hour time point in the shESE-1 or shCtr knockdown lanes (Fig. 5A, lanes 2-3). At the 72-hour time point there is minimal DNA smearing in the empty vector (Fig. 5A, lane 4) and shCtr (Fig. 5A, lane 5) controls, and even less smearing in the shESE-1 treated cells (Fig. 5A, lane 5). As a positive control, MCF-7 cells were treated with Trail plus cycloheximide, and these cells display robust DNA smearing evincing apoptosis (Fig. 5A, lane 7). To further investigate apoptosis as a biological response to ESE-1 knockdown, we analyzed caspase 3 and/or 7 activation, using a luminescent enzyme activity assay (CaspaseGlo 3/7 Assay, Promega). With the data set to 1 for untransfected controls, these results show that there is no change in caspase 3/7 activity in shESE-1 transfected cells, compared to empty vector and shCtr transfected cells, at either the 48 or 72 hour time points (Figs. 5B and C). Having excluded apoptosis as the cellular mechanism responsible for the shESE-1-induced inhibition of colony formation, we next sought to determine whether shESE-1 affected MCF-7 cellular proliferation.

Reversion of the Transformed Phenotype is due to shESE-1-mediated Inhibition of MCF-7 Cell Proliferation

The control of cellular proliferation is a key mechanism in the prevention of tumorgenicity and malignancy. ESE-1 has been shown to regulate promoter activity of the *Her2/neu* and *TGF-βRII* genes (12, 23-26). These two plasma membrane receptors contribute to the regulation of breast cancer cell growth and proliferation. ESE-1's transcriptional regulation of these receptors suggests that it has an important role in controlling cellular proliferation. In order to confirm ESE-1's role in maintaining cellular proliferation in MCF-7 transformed cells, MCF-7 cells were transiently transfected with shCtr or shESE-1, and cellular proliferation was determined at 2-, 4- and 6-days post-transfection by counting total viable cells (Fig. 6A) and 6-days post-transfection using an MTS assay (Fig. 6B). A representative total cell proliferation study, performed in duplicate, shows that shESE-1 cells display significantly reduced proliferation at each time point (Fig. 6A). At the start of the study, 50,000 cells were plated, with shCtr cells showing 82,500, 105,000 and 260,000 at 2- 4- and 6-days post-transfection and the shESE-1 cells showing 31,000, 23,500 and 89,000 at the same time points. These results reveal a 62%, 78% and 66% reduction in cell proliferation at 2- 4- and 6-days, respectively, in the shESE-1 knockdown cells compared to shCtr cells (Fig. 6A). Similar results were obtained using an MTS proliferation assay, which allowed us to perform 8 replicates in a 96-well format, and showed a 1.6 fold (or ~62%) reduction in MTS absorbance at 6-days post-transfection in the shESE-1 transiently transfected MCF-7 cells, compared to shCtr control cells (Fig. 6B). Again, because we had to use transient transfection, the inhibitory effects of shESE-1 on MCF-7 cell proliferation are likely to be underestimated, since not all MCF-7 cells were transfected with shESE-1 DNA and even transfected cells begin to regain endogenous ESE-1 expression by 5 days post-transfection. These data

further demonstrate that ESE-1 is required for optimal MCF-7 cellular proliferation and reveal the mechanism by which ESE-1 contributes to the transformed phenotype.

Serum starvation extinguishes ESE-1 protein expression in MCF-7 cells

Having shown that MCF-7 cells express endogenous ESE-1 protein (Fig. 1) and that ESE-1 expression is required to maintain the MCF-7 transformed phenotype by controlling cellular proliferation (Figs. 4, 5 and 8), we sought to determine whether ESE-1 protein production was growth factor dependent. This question is relevant because ESE-1 protein regulates *HER2/Neu* promoter activity (12, 15) and HER2/Neu has been shown to activate the *ESE-1* promoter (11), suggesting that growth factors present in serum may play a critical role in regulating *ESE-1* gene expression and ESE-1-mediated mammary cell proliferation. Here, we grew MCF-7 cells in either complete (10% FBS) or in serum-depleted (0.1% FBS) media for 6, 18 and 36 hrs, and then probed for ESE-1 and tubulin protein expression by Western blot analysis of whole cell extracts. As shown in Fig. 6C, ESE-1 protein appears to increase slightly at the 6 hr post-starvation time-point, only to decrease progressively at the 18 and 36 hr post-starvation time-points, compared to complete media controls. Quantitation of the 36 hr post-starvation time-point revealed a 90% reduction of ESE-1 protein expression, when normalized to tubulin and compared to the 36 hr complete media control. The Western blot for tubulin reveals that protein loading was equivalent in all lanes. These data raise the interesting possibility that reduction in MCF-7 cell proliferation due to serum starvation may be due, at least in part, to reduced ESE-1 protein expression.

Discussion

The ETS transcription factor family is known to play a significant role in many cancers, with aberrant expression of ESE-1 being correlated in nearly 50% of early human breast tumors. In addition, ectopically expressed ESE-1 has been shown to impart the transformed phenotype on MCF-12A and MCF-10A nontransformed mammary epithelial cell lines (15, 20-22, 27). While dominant-negative ETS approaches, which interfere with multiple ETS factors, have reversed the transformed phenotype in several breast cancer cell lines (NmuMG, MMT and BT20) (18, 19), here we show that the knockdown of a single ETS factor, ESE-1, has the same effect in MCF-7 breast cancer cells. Importantly, we show that ESE-1 is required to maintain the transformed phenotype in MCF-7 breast cancer cells, since shRNA-mediated ablation of endogenous ESE-1 protein resulted in decreased colony formation and anchorage-independent growth (Fig. 4). Similar results were obtained with ZR-75 cells, with shESE-1 resulting in decreased colony formation (Fig. 4A) and anchorage-independent growth (data not shown). Furthermore, mechanistic studies, using two separate approaches to measure apoptosis and proliferation, revealed that ESE-1 does not modulate apoptosis, but rather is required for cancer cell proliferation (Figs. 5 & 6A and B). Finally, we also demonstrate that serum is required to maintain ESE-1 protein production (Fig. 6C), raising the interesting possibility that reduced ESE-1 protein expression occurring upon serum starvation likely contributes to reduced MCF-7 cell proliferation noted in these conditions. Taken together, this paper contributes novel insights to our understanding of the critical role of ESE-1 in maintaining cell transformation of mammary epithelial cells via regulation of cellular proliferation.

Protein expression and characterization studies of ESE-1 in breast cancer cell lines and tissues have been limited, in large part due to the lack of highly-specific anti-

ESE-1 antibodies. Similar to our results shown here, most studies using Western blot analysis of breast cancer cell lines have reported that MCF-10A, MCF-12A and MDA-MB-231 cells typically do not express ESE-1, whereas MCF-7, ZR-75 and T47D cells do express it (25, 27-29). However, unlike our results, one group reported ESE-1 protein expression in MCF-12A cells (25), and another group reported that T47D cells fail to express ESE-1 protein (29). We have consistently not been able to detect ESE-1 in MCF-12A cells, using PCR to detect mRNA (Fig. 1B) (21) and Western blot, IHC or ICC analyses to detect protein (Figs. 1A and 2) (20). Thus, one possibility to explain this discrepancy is that MCF-12A cells expressing ESE-1 have undergone spontaneous transformation, suggesting that MCF-12A cells may be poised to be easily transformed, requiring the use of low passage cells grown in defined media to avoid transformation and possible activation of ESE-1 expression (21, 30). Another discrepancy is that we show the level of ESE-1 protein to be MCF-7 = T47D > ZR-75, whereas previous reports show MCF-7 > ZR-75-1 >> T47D (25, 28, 29). This disparity could be explained by slight differences in growth conditions or subclone characteristics. Indeed, we have found ESE-1 expression to vary in certain T47D sublines (data not shown). Finally, we show that ESE-1 mRNA levels generally correlate with protein data in the mammary cell lines studied here, with T47D and MDA-231 showing a slight discordance between the mRNA and protein levels (Fig. 1).

ESE-1 contains several functional NLS and NES signals [(20, 31) and Prescott and Gutierrez-Hartmann, unpublished data)], suggesting that ESE-1 shuttles between the nuclear and cytoplasmic compartments. We and others have demonstrated that transient transfection and adenoviral transduction studies consistently show nuclear localization of ESE-1/Elf-3 and we have used such transient ESE-1 expression assays to map the transcriptional properties and sites of co-factor interactions of ESE-1/Elf-3 as a

nuclear effector (15, 20, 28, 31-34). Here, using MAb405 in ICC studies, we show that endogenous ESE-1 is detected in the nucleus in MCF-7, T47D and ZR-75 human mammary epithelial cancer cells (Fig. 2). However, several IHC studies of endogenous ESE-1 show cytoplasmic ± nuclear localization in T47D and ZR-75 cell lines (27, 35), and mammary, retinal pigment and synovial epithelial cells (20, 25, 28, 36-38). Thus, the differences in subcellular localization of ESE-1 noted in these various studies may be due to the different experimental conditions, different antibodies used for detection, and different types of assays: eg, IHC vs ICC, transient vs stable, ectopic vs endogenous, *in vitro* vs *in vivo*, and nontransformed vs transformed. Nevertheless, in combination, these studies reveal that ESE-1 can be detected in the nuclear and/or cytoplasmic compartments.

Previously, we have shown that stably expressed HA-ESE-1 or GFP-ESE-1 imposes the transformed phenotype on nontransfromed, ESE-1-negative MCF-10A and MCF-12A cells (20, 21), and that cytoplasmic localization of a 40-AA SAR domain is necessary and sufficient to mediate this effect (20). Consistent with this conclusion, PAK-1-mediated phosphorylation of the SAR domain of exogenous ESE-1 in the cytoplasm modulates stability and transformation potency of ESE-1 in ZR-75 cells (27). Here we show that ESE-1 knockdown in transformed MCF-7 and ZR-75 cells that express endogenous ESE-1, reverses their transformed properties. Moreover, we demonstrate, by ICC, that ESE-1 is localized to the nucleus in MCF-7, T47D and ZR-75 cells, indicating that the ability of ESE-1 to maintain the transformed phenotype requires its role as a nuclear transcription factor. Of note, we have previously demonstrated that GFP-ESE-1 targeted to the nucleus of nontransformed MCF-10A and MCF-12A cells induces apoptosis, whereas transformed T47D and Sk-Br-3 cells tolerate nuclear expression of exogenous ESE-1 without inducing apoptosis, possibly because anti-

apoptotic pathways have been up-regulated in these transformed cells (20). Taken together, these data suggest that ESE-1 initiates transformation in ESE-1-negative mammary epithelial cells via a cytoplasmic- and PAK-1-dependent mechanism, but once fully transformed, these cells require the nuclear transcription properties of ESE-1 to maintain the transformed phenotype.

Ets factors have been associated with a number of malignancies, particularly in the mammary gland and prostate (1). The large redundancy of Ets factor genes in humans (~27) has required dominant-negative Ets approaches, whereby function of multiple Ets factors is blocked. This dominant-negative method has validated that Ets factors are required for mammary cell transformation (1); however, this study failed to identify whether an individual Ets factor is sufficient to maintain the transformed phenotype. A key significance of this report is that we use shRNA technology to knockdown a single Ets factor, ESE-1, to demonstrate that it is necessary and sufficient to maintain the transformed phenotype in MCF-7 and ZR-75 human mammary epithelial cancer cells. Thus, the clinical significance is that ESE-1 provides a novel single-molecule target for breast cancer therapy. The ESE-1 19-nt target sequence selected maps to the DBD. While BLAST searches with this 19-nt sequence only identified ESE-1, manual analyses of ETS DBD sequences revealed that ETS-1 and ETS-2 showed the closest similarity, with only 4/19 matches. While this level of mismatches is very unlikely to allow the ESE-1 shRNA to target ETS-1 and/or ETS-2, we nevertheless performed Western blot analysis and demonstrated specificity for knockdown of ESE-1 (Fig. 3B). Relevant to this report is the *Ets2* knock-out study revealing that transgenic mice expressing mammary-targeted polyoma virus middle T oncogene crossed to heterozygous female mice carrying only one wild-type *Ets2* allele, resulted in smaller tumors compared to crosses with wild-type controls (39). Further analysis revealed that

Ets2 functions in the stromal compartment to regulate mammary epithelial tumor growth (40). The transgenic papers show a ~50% reduction in mouse mammary tumor size, due to the stromal effects of Ets2, whereas we show an ~80-90% reduction in colony formation in MCF-7 and ZR-75 cells stably expressing shESE-1 (Fig. 4A and B) and a 57% reduction in soft agar colony numbers in MCF-7 cells transiently expressing shESE-1 (Fig. 4C and D). Indeed, the colonies growing in G418-selected MCF-7 cells, with stable knockdown of ESE-1, consistently failed to expand, underscoring that ESE-1, when more fully ablated, has a dominant growth and/or survival effect in mammary epithelial cells. Taken together, these data reveal that specific, individual Ets factors have critical roles in mammary carcinogenesis, but in distinct tissue compartments, with Ets2 functioning in the stroma and ESE-1 functioning in the mammary epithelial cell.

Having demonstrated that ESE-1 is required to maintain the transformed phenotype of MCF-7 cells, we proceeded to mechanistic studies evaluating whether ESE-1 knockdown induced apoptosis or affected cell proliferation. Specifically, we used both DNA laddering and caspase 3/7 cleavage assays, in order to rigorously assess whether ESE-1 knockdown caused apoptosis, and the data show that ESE-1 knockdown does not induce MCF-7 apoptosis (Fig. 5). Instead, two separate experimental approaches, total cell counts and MTS assay, revealed that ESE-1 knockdown has a key effect on MCF-7 cell proliferation (Fig. 6A and B). Again, these studies required transient transfection of shESE-1 in order to expand the cells over the 6-day assay period, and despite the fact that not all cells take up plasmid DNA, shESE-1 knockdown resulted in a 73% and 46% inhibition of proliferation in the total cell count and MTS assays, respectively, at 6 days (Fig. 6A and B). Certain ETS proteins control cell proliferation by inducing growth factors and/or their receptors, with ESE-1 shown to trans-activate HER2/neu (1, 5, 15, 34, 41, 42). Moreover, heregulin/HER2, EGF and

serum induce ESE-1 promoter activity in Sk-Br-3 human breast cancer cells, and lactogenic competency induced in murine HC-11 mammary cells by dexamethasone, insulin and prolactin, also increased ESE-1 mRNA expression (25). Consistent with these findings, here we show that removal of serum growth factors significantly diminishes ESE-1 protein production (Fig. 6C). These data further suggest that a forward autocrine regulatory loop may exist between ESE-1 and growth factors, such as HER2/neu, and that such a regulatory loop contributes to the regulation of cell proliferation, and ultimately transformation, by ESE-1.

Materials and methods

Cell Lines

MCF-7, T47D, MDA-231, MCF-10A, and MCF-12A cells were maintained as described previously (15, 21). ZR-75 cells were cultured in minimum essential medium (MEM) supplemented with 5% FBS, 1x non-essential amino acids, and 1 μM insulin.

shRNA Constructs and Transfection

ESE-1 shRNA and shCtr oligonucleotides were designed using Oligoengine (Seattle, WA). The oligonucleotide targeting ESE-1 (shESE-1) is follows: forward oligo 5' ACAGCAACATGACCTACGATTCAAGAGATCGTAGGTCATGTTGCTGT-reverse oligo 5' ACAGCAACATGACCTACGATCTCTTGAATCGTAGGTCATGTTGCTGT. The ESE-1 negative control shRNA oligonucleotide is as follows: forward oligo 5' GCTCAACGAGGGCCTCATGTTCAAGAGACATGAGGCCCTCGTTGAGC reverse oligo 5' GCTCAACGAGGGCCTCATGTCTCTTGAACATGAGGCCCTCGTTGAGC. This negative control (shCtr) was originally designed to target ESE-1 expression, but since qRT-PCR and Western blot studies showed it failed to inhibit ESE-1 expression, we

used it as a negative control. The ESE-1 shRNA and shCtr were cloned into pSuper at the *Bgl*II and *Hind*III restriction sites. Each pSuper shRNA vector was co-transfected along with pEGFP-C3 at a 10 to 1 ratio (pSuper:pEGFP) into MCF-7 or ZR-75 cells using Qiagen Effectene, with a 12 to 1 ratio of DNA to Effectene. Cell lysates were generated two days post-transfection, unless otherwise noted.

Western Blotting

Western blot analysis was performed essentially as described previously (15, 20), but cells lysis was completed by re-suspending them and incubating them on ice for 25 min in 0.1% NP-40, 50 mM Hepes pH 7.2, 250 mM, 2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM benzamidine, 1 mM DTT, 25 μg/ml aprotinin, 25 μg/ml trypsin inhibitor, 25 μg/ml leupeptin, and 25 μg/ml β-glycerophosphate. Cells were then vortexed in four 30 second intervals, followed by a 15 min centrifugation at 13 000 g at 4°C, and the supernatant was collected. Protein concentrations were determined using a Bio-Rad detergent-compatible protein assay.

Quantitative RT-PCR

The qRT-PCR study was performed as described (43). Plasmid pEGFP-ESE-1 (20) was used to generate sense-strand ESE-1 RNA, which was then used as an absolute standard for qRT-PCR. Primers and probes for ESE-1 were designed using the Prism 7700 sequence detection software (Primer Express, Perkin-Elmer Corp./Applied Biosystems (PE ABI), Foster City, CA), resulting in the following oligos: Forward ESE-1 Primer: (1109)AGCCGGGCCATGAGGTAC(1126); Reverse Primer: (1173)ACGAGTCGCCGGCCAT(1158); and TaqMan Probe: (1131)ACAAACGGGAGATCCTGGAACGGG(1154). Total RNA was prepared from cells using RNA Stat-60 (Tel-Test “B”, Inc.) and qRT-PCR was performed using an ABI

PRISM 7700 Sequence detector (PE ABI), with PCR reactions monitored in real time. Reverse transcription, PCR conditions, real-time data acquisition and analyses were performed as described (43). ESE-1 mRNA was normalized to the amount of 18s rRNA (PE ABI, P/N 4308310) in each sample.

Immunocytochemistry (ICC)

Cells (50 000) were plated directly onto glass cover slip in a 12 well tissue culture plate. Two days post-plating, cells were fixed with 2% paraformaldehyde (PFA) in 1x PBS for 20-25 min at room temperature (RT), followed by three 5 min washes in 1x PBS. Cells were permeabilized at RT with 0.5% Triton X-100 in 1x PBS for 10 minutes, followed by three 10 min washes in 100 mM glycine in 1x PBS. Permeabilized cells were blocked in a 1x PBS, 0.5% Tween-20, 10% goat serum, 0.05% bovine serum albumin (BSA) blocking buffer at RT within a moisture chamber for 1-2 h. Cells probed for ESE-1 were incubated in 1:500 antibody:blocking buffer overnight at 4°C in a moisture chamber. To measure auto-fluorescence, cells were incubated overnight at 4°C with blocking buffer alone. shRNA tranfected cells underwent the same procedure at 48 h post-transfection.

Colony Formation Assays

MCF-7 and ZR-75 cells were transfected in suspension with Effectene, as described above, using 250 000 cells and 800:80 ng of shRNA:pEGFP-C3 in sterile 1.5 ml microfuge tube per transfection. Transfected cells (500 000 MCF-7 and 250 000 ZR-75) were then seeded on 60 mm plates, and 48 h post-transfection, cells were treated with 500 μg/ml G418 (Gibco) for 14 days. On day fourteen, cells were fixed with 2% PFA for 30 min, washed twice in 1x PBS, and then stained for 30 seconds with crystal violet and washed for 1 min in ddH_2O. Colonies were counted by direct visual analysis.

Colony Formation in Soft Agar

Anchorage independent growth was determined by assaying colony formation in soft agar. MCF-7 cells were transfected and 50 000 cells were seeded by re-suspending cells in 1.5 ml of 0.3% agar noble (BD Scientific Difco) in DMEM, and plated in 6 well plates containing a 1.5 ml 0.6% agar noble in DMEM base layer. Cells were fed every three days up to 14 days. Colonies were stained overnight at 37°C with 150 μl of 1M nitroblue tetrazolium chloride (Amresco) in 1x PBS and quantitated using Metamorph imaging software, set to a colony threshold size of 150-250 microns.

Apoptosis Assay

MCF-7 cells were transiently transfected with empty vector, shCtr or shESE-1 DNA (~$1x10^6$ cells per DNA) and used for both the DNA laddering and caspase 3/7 assays. For the DNA laddering, transfected cells were harvested 24 h post-transfection, cells were counted and genomic DNA was isolated from 100 000 cells. DNA was analyzed on a 1% agarose gel stained with ethidium bromide. Positive control cells were treated with 75 μg/ml of TRAIL plus 1 μg/ml of cycloheximide. For the Caspase-Glo 3/7 assay transfected MCF-7 cells were collected 24 h post-transfection with 1x PBS/EDTA, counted and 3 000 transfected cells were plated in a 96-well plate. Caspase 3/7 reagent was added to cells at 48 h and 72 h time points, and assays were performed as described by the manufacture (CaspaseGlo 3/7 Assay, Promega).

Proliferation assays

Total cell count proliferation assays were conducted by plating 50 000 cells/well in a 12-well plate, and transfecting these cells with pEGFP-C3 and the indicated shRNA. Cells were harvested with 1x PBS/EDTA on days 2, 4, and 6 post-transfection, and viable cells were counted using the Vi-Cell counter (Beckman Coulter). MTS proliferation

assays were conducted by transfecting MCF-7 cells with pEGFP-C3 and the indicated shRNA, and harvested 24 h later by suspension into 1x PBS-EDTA. Viable cells were counted by staining cells with Trypan blue and using a Vi-Cell-cell viability analyzer (Beckman Coulter), with 5 000 cells/well plated in a 96-well plate. Six days later, cells were counted using the MTS proliferation assay, as described by manufacturer (Promega).

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Titles and Legends to Figures

Figure 1. **ESE-1 protein is expressed in several human breast cancer cell lines**. A.) Western blot of whole cell extracts (100 μg) generated from MCF-10A (lane 1), MCF-12A (lane 2), MCF-7 (lane 3), T47D (lane 4), ZR-75 (lane 5) and MDA-231 (lane 6) cells, probed with anti-ESE-1 mAB405 antibody (1:1000 dilution) and anti-tubulin mouse monoclonal antibody (1:10 000 dilution, CP06, Calbiochem). B.) Quantitative RT-PCR analysis of endogenous ESE-1 in MCF-10A, MCF-12A, MCF-7, T47D, ZR-75 and MDA-231 human mammary cell lines. Total RNA (1 μg) was generated from each cell line and used for qRT-PCR analysis (ABI PRISM 7700, PE ABI). ESE-1 mRNA in each sample calculated relative to absolute ESE-1 values that were derived from a standard curve, using a known amount of sense-strand ESE-1 RNA, and then normalized to total input RNA, using 18S rRNA measured from 1 ng total RNA.

Figure 2. **ESE-1 is localized to the nucleus in human breast cancer cell lines.** The top row are confocal images of ZR-75, MCF-7, T47D, MCF-12A, MCF-10A and MDA-231 mammary epithelial cells probed with the anti-ESE-1 mAB405 antibody (1:500 dilution), followed by a Cy3-conjugated goat anti-mouse secondary antibody (1:200 dilution). ZR-75, MCF-7, T47D show a positive (red) signal and are grouped in the left panel, whereas MCF-12A, MCF-10A and MDA-231 are negative and are grouped in the right panel. The second row from the top shows confocal images of the same cells stained with DAPI (100 ng/ml; blue nuclei), and the third row shows the merge of the top two rows. Shown at the bottom are the negative controls, depicting confocal images of these same cells probed with blocking solution and the Cy3-conjugated goat anti-mouse secondary antibody (1:200 dilution), but omitting the anti-ESE-1 mAb. The bottom row shows confocal images of DAPI-stained, negative control cells.

Figure 3. shESE-1 knocks down endogenous ESE-1 protein in ZR-75 and MCF-7 cells in a specific and prolonged manner. A.) Time-course of ESE-1 knockdown after transient shESE-1 transfection in ZR-75 cells. ZR-75 cells (~60% confluent, 10 cm plate) were transiently transfected with shESE-1 (6.5:0.65 μg; shESE1:pEGFP-C3), and lysates were harvested on days 2, 3, 4 and 5 post-transfection. The negative control lysate was generated 2 days after transfecting ZR-75 cells with pSuper/empty vector. Western blot of whole cell lysates (100 μg) using the anti-ESE-1 mAB405 (1:1000) is shown in the top panel, with the same blot stripped and re-probed with tubulin antibody (1:10 000) and shown in the bottom panel. B.) Specificity of shESE-1 knockdown in MCF-7 cells. MCF-7 cells were transiently transfected with shCtr or shESE-1, harvested 48 h post-transfection and Western blot of 100 μg of whole cell extract with anti-ESE-1 mAB405 (1:1000) shown in the top panel. The middle panel shows a Western blot of 35 μg of the same MCF-7 whole cell extracts, probed with anti-Ets1/Ets2 rabbit polyclonal antibody (1:10 000 dilution, Santa Cruz, c-275). The PVDF membrane shown in the top panel was washed and re-probed with anti-tubulin antibody (1:10 000), and the resultant Western blot is shown in the bottom panel. C.) Arbitrary densitometry units (ADU) measuring the ESE-1 densitometry signal normalized against tubulin, with the shCtr signal set to 1.

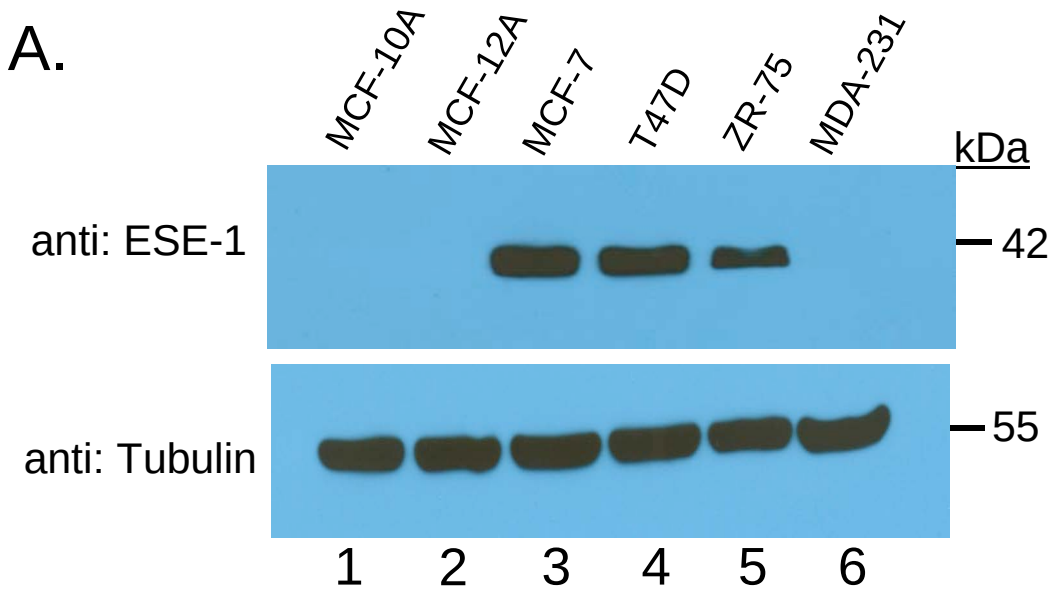
Figure 4. ESE-1 knockdown abrogates ZR-75 and MCF-7 colony formation and knockdown diminishes soft agar colony formation in MCF-7 cells. A.) ZR-75 and MCF-7 colonies stained with Crystal violet. ZR-75 cells (top panel) or MCF-7 cells (bottom panel) were transfected with shCtr or shESE-1 (using different DNA preparations for the ZR-75 and MCF-7 cells), selected for 14 days with 500 ug/ml of G418, and resultant colonies were stained with crystal violet. B.) Direct visual

quantitation of ZR-75 (dark bars) and MCF-7 (grey bars) colonies. Cells were transfected in triplicate and selected with G418, as above, and the resultant colonies were counted by visual inspection. The shESE-1-mediated colony reduction in ZR-75 and MCF-7 cells is statistically significant to p=0.0006 and p=0.008, respectively, using the Student's t-test. C.) A digital image of NBT-stained soft agar colonies generated from MCF-7 cells transiently transfected with shCtr or shESE-1. D.) Quantitation of soft agar colonies. MCF-7 cells were transiently transfected with shCtr or shESE-1, plated in sextuplicate and colonies quantitated using the Metamorph imaging software (threshold 150-250 microns). shESE-1 mediated a 56% reduction in MCF-7 soft agar colony formation, which was significant to a p=0.002 value, using the Student's t-test.

Figure 5. Knockdown of ESE-1 does not induce DNA laddering or Caspase 3/7 activity. MCF-7 cells were transiently transfected with empty vector (lanes 1 & 4), shESE-1 (lanes 2 & 5) and shCtr (lanes 3 & 6), and genomic DNA was isolated at 48 and 72 h post-transfection. Isolated DNA (5 μg) was separated on a 1% agarose gel and stained with ethidium bromide. Positive control MCF-7 cells (lane 7) were treated with 75 μg/ml of trail plus 1 μg/ml of cycloheximide for 24 h, and DNA (5 μg) was isolated and analyzed as above. B. and C.) For the caspase assays MCF-7 cells were transiently transfected with empty, shESE-1 and shCtr DNAs in triplicate, and caspase 3/7 activity was measured at 48 h (B) and 72 h (C) post-transfection. The capsase 3/7 activity of untransfected controls at 48 and 72 h was set to 1, and the caspase 3/7 activity of transfected cells was normalized to the untransfected value and expressed as fold-change.

Figure 6. Proliferation of MCF-7 cells is reduced with knockdown of ESE-1. Also, serum starvation of MCF-7 cells reduces endogenous ESE-1 protein expression

A.) Total cell counts over 6 days. MCF-7 cells were transiently transfected with shCtr (diamonds) or shESE-1 (squares), and 24 h post-transfection cells were counted and 50 000 seeded at time 0. Cells were collected on days 2, 4, and 6 with 1 x PBS/EDTA and counted using the Beckman Coulter Vi-cell. B.) MTS proliferation assay at 6 days. MCF-7 cells were transiently transfected with shCtr or shESE-1, replicated eight times, and 24 h post-transfection cells were counted and 5 000 cells were plated at time 0. Cells were grown for 6 days, harvested and MTS measured at 490 nm, as in Methods. The difference in shCtr vs shESE-1 was significant to p=1.67E-07, using the Student's t-test. C.) MCF-7 cells were plated in either 10% serum (+) or 0.1 % serum (-) media and harvested at 6 h (lanes 1, 2), 18 h (lanes 3, 4) and 36 h (lanes 5, 6) later. The top panel shows a Western blot of MCF-7 whole cell extracts (100 μg) probed with anti-ESE-1 mAB405 antibody (1:1000 dilution). The bottom panel shows the same blot stripped and re-probed with anti-tubulin antibody (1:10 000).



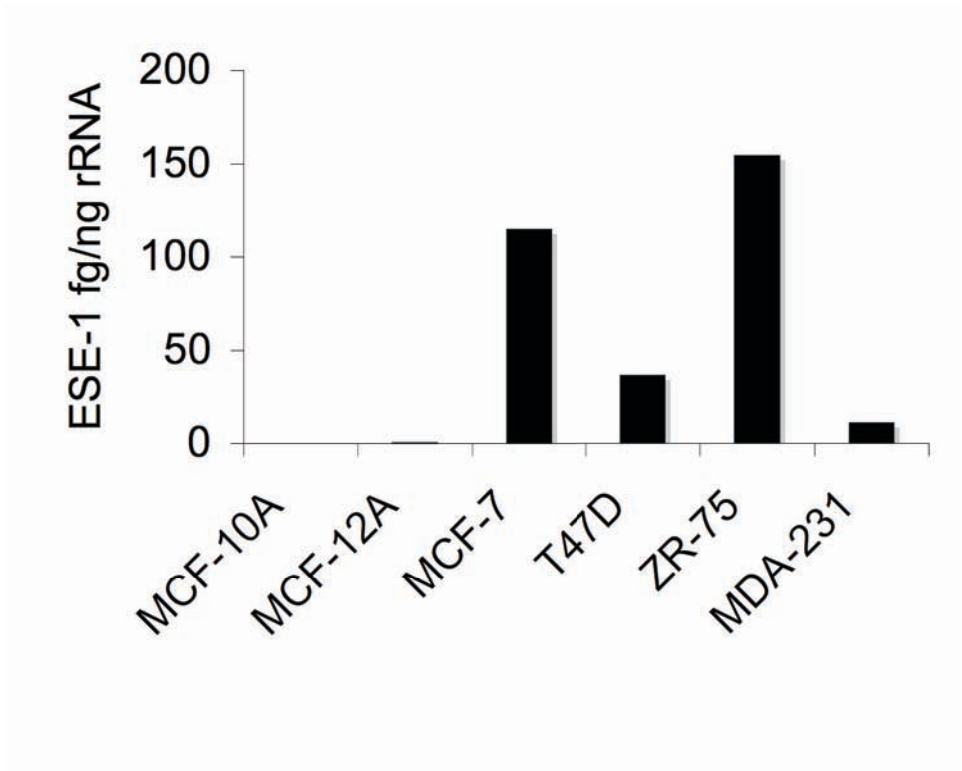


Fig. 1

Walker et al

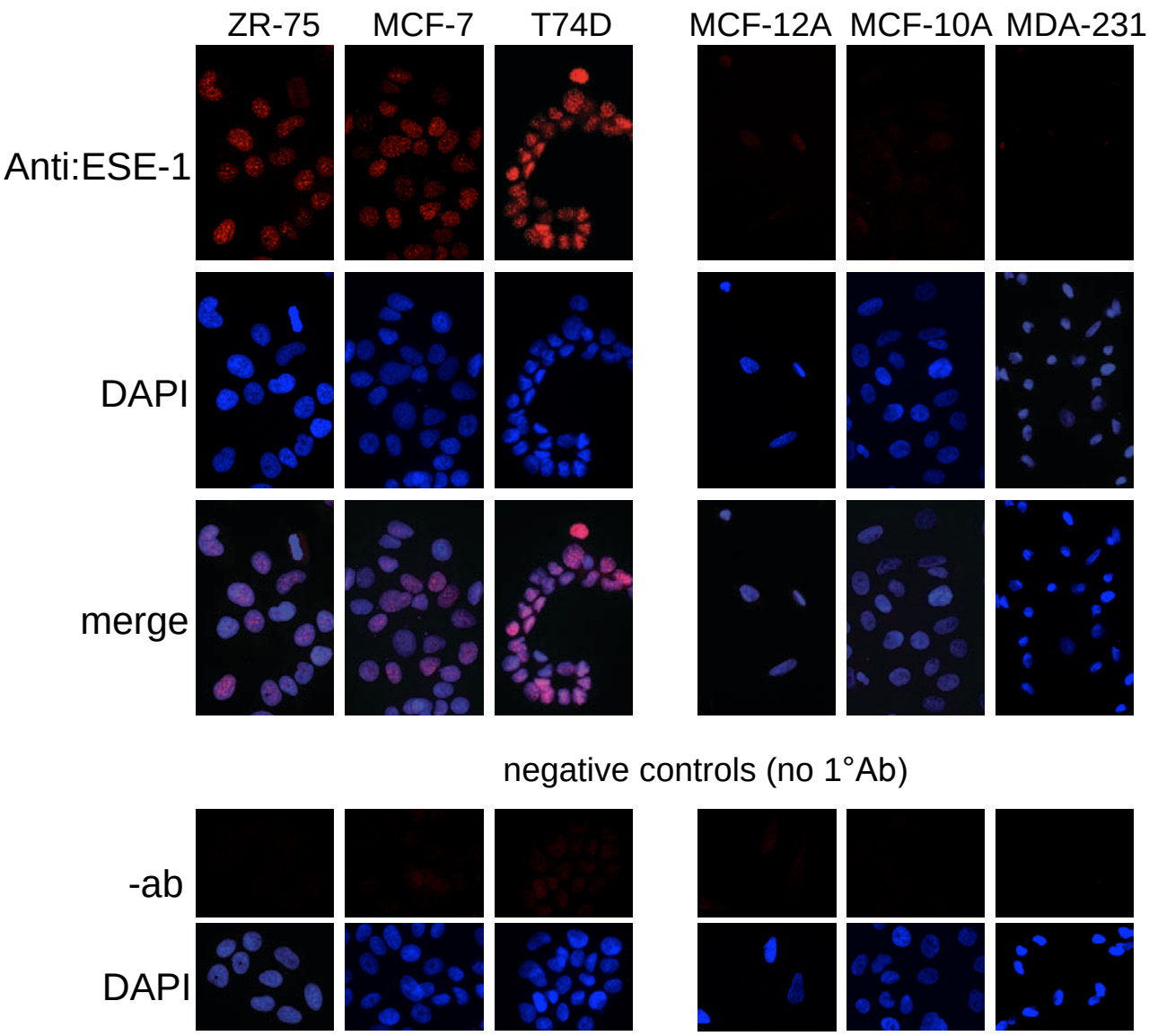


Fig. 2

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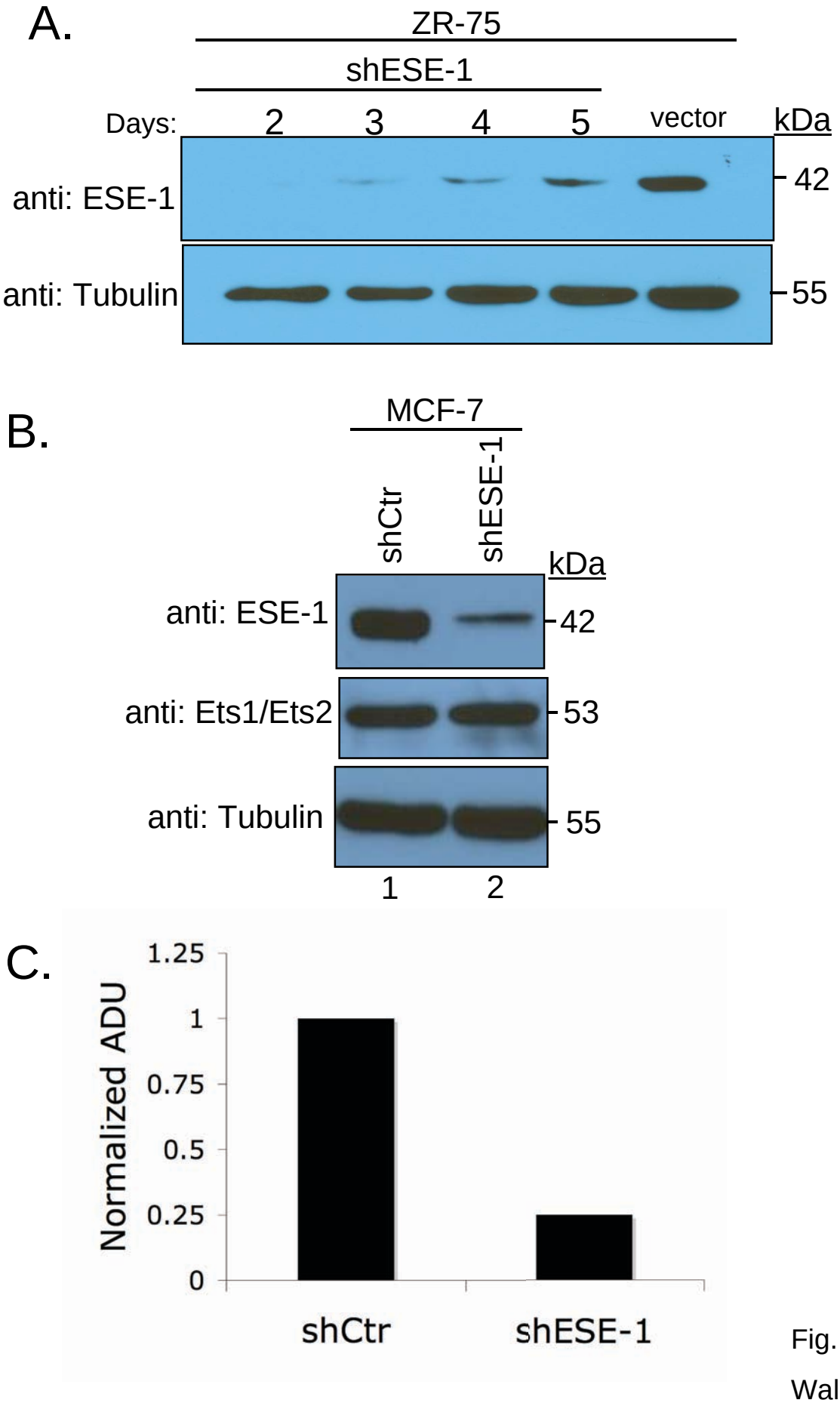

Fig. 3

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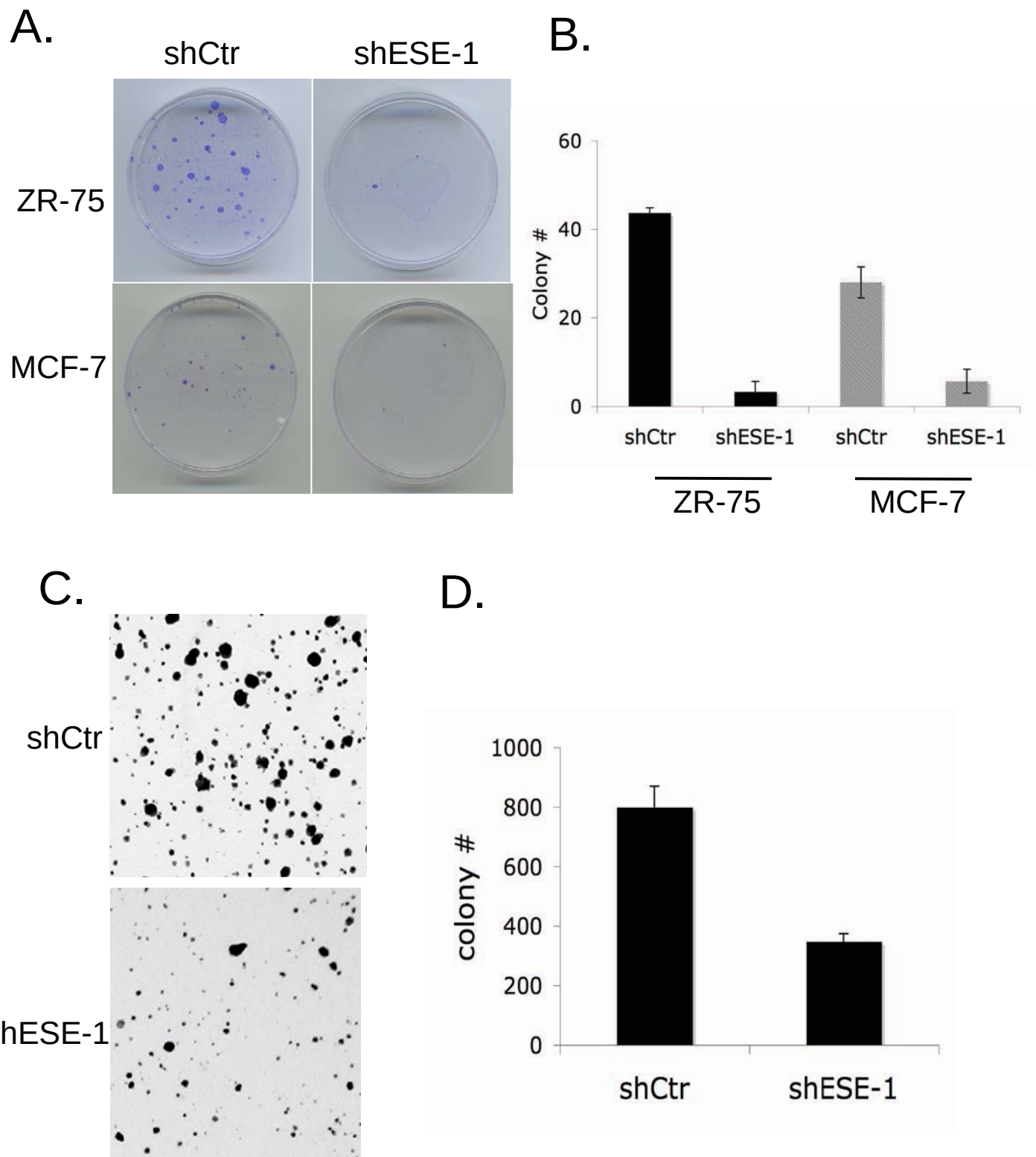

Fig. 4

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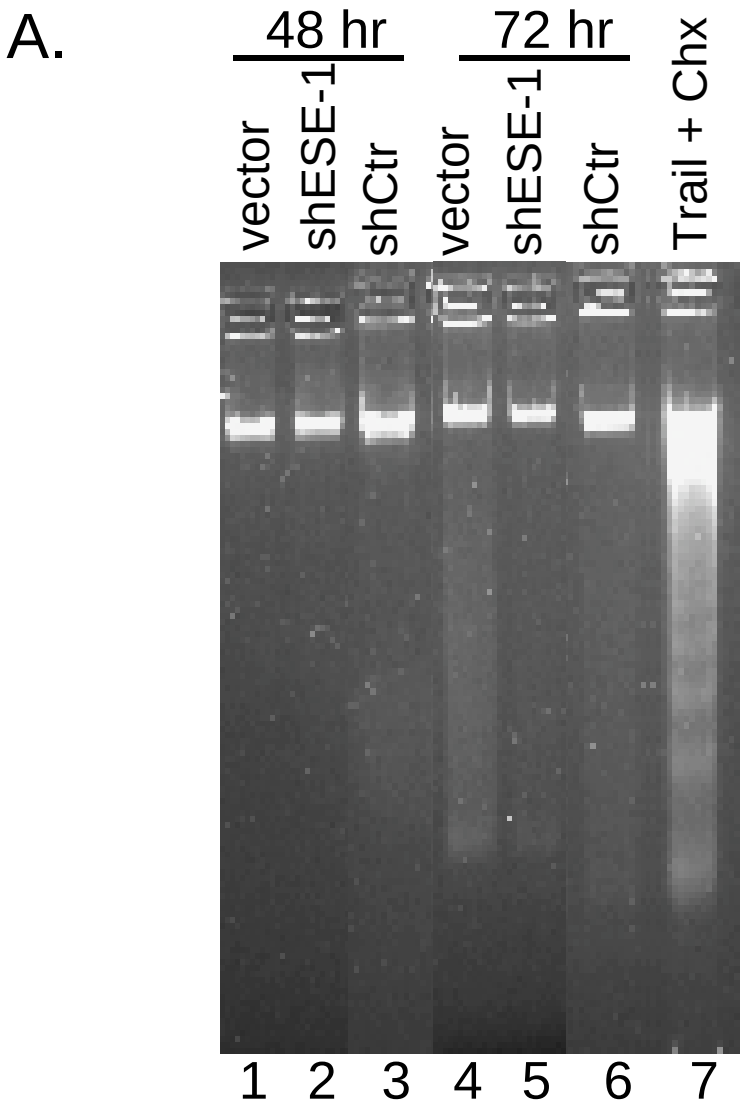
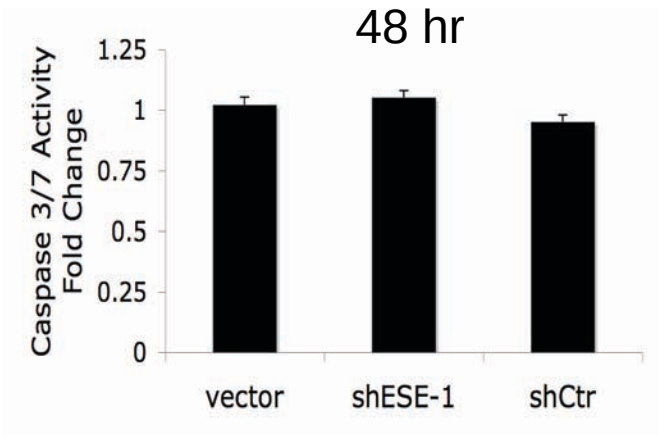




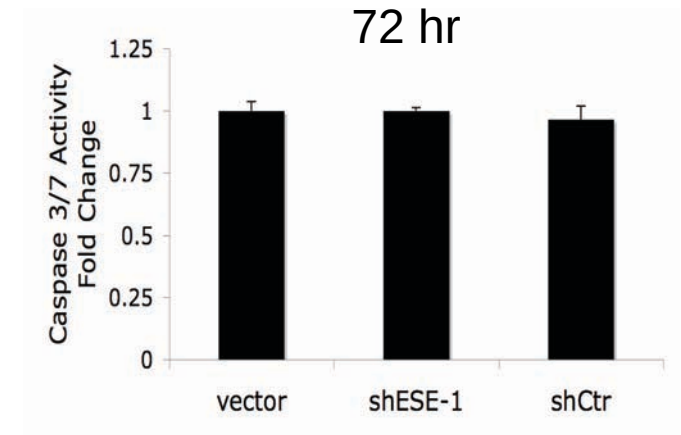
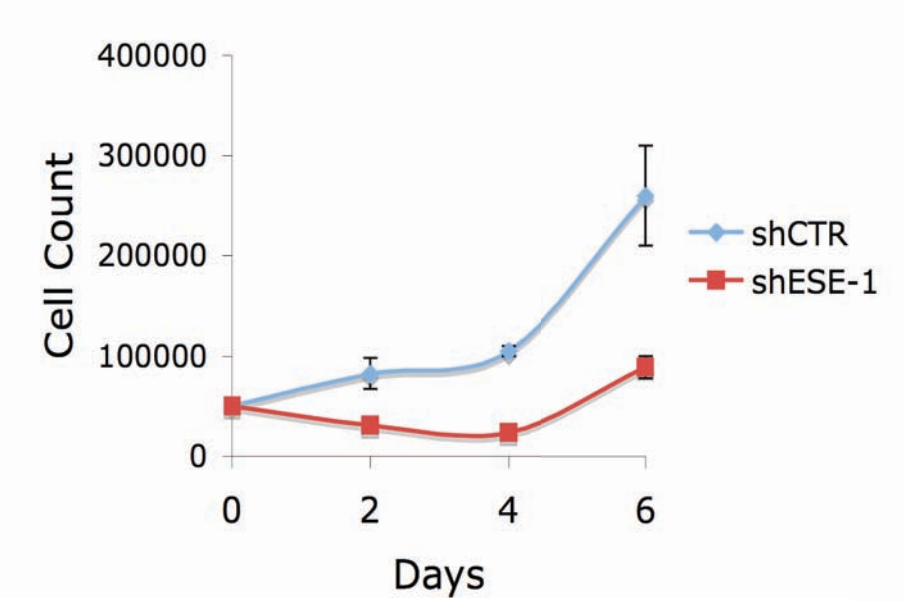


Fig. 5

Walker et al

A.



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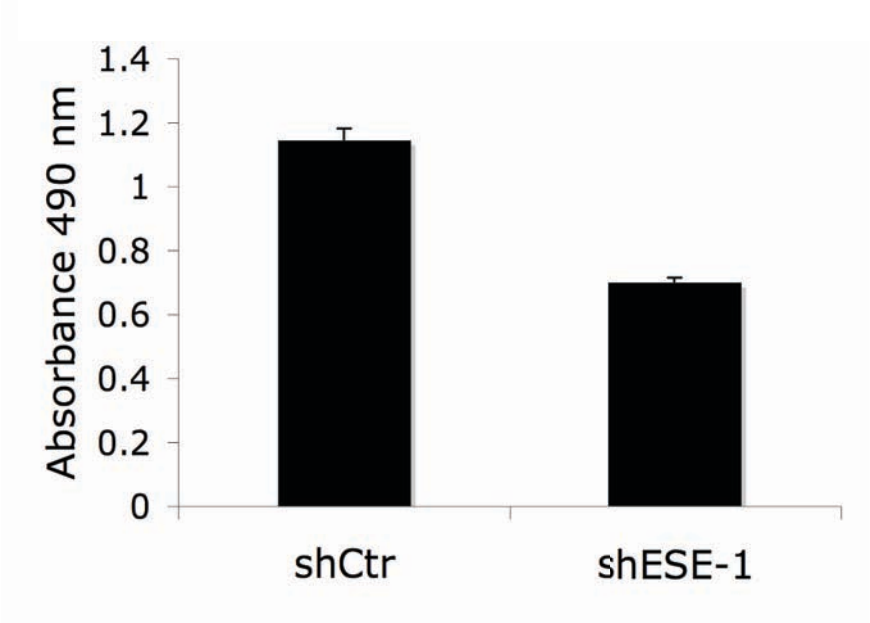

C.

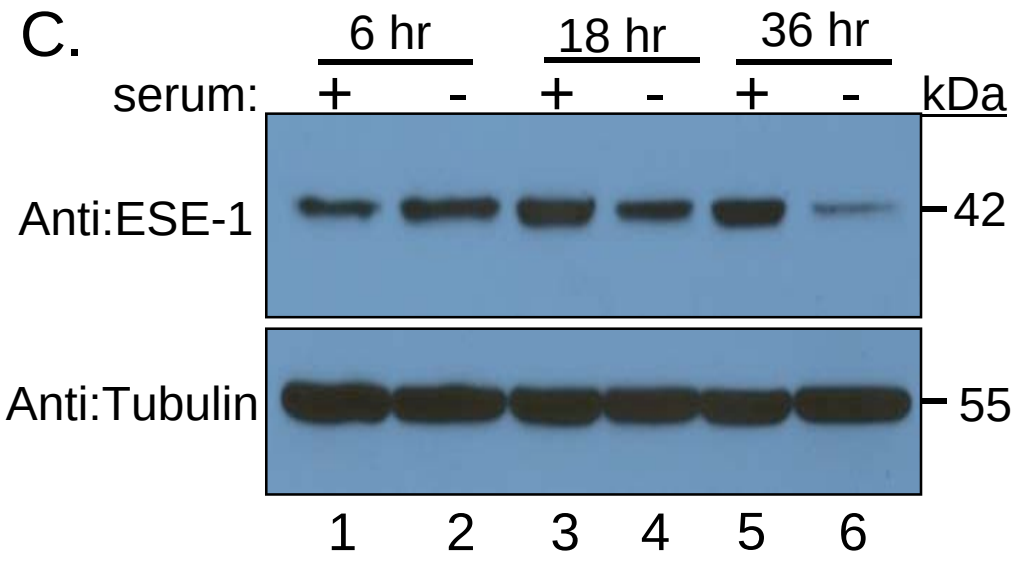

Fig. 6

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